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(54) Title: METHODS AND REAGENTS FOR THE IDENTIFICATION AND REGULATION OF SENESCENCE-RELATED GENES

(57) Abstract

Identification of senescence-related genes can be accomplished by comparing mRNA expression between young and senescent cells. Probes complementary to such genes can be used to detect senescent cells and distinguish between young and senescent cells as well as in screens to identify compounds that alter expression levels of senescence-related genes.

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Description

Methods and Reagents for the Identification and Regulation of Senescence-Related Genes

Background of the Invention

Field of the Invention

The present invention relates to the fields of molecular biology, gerontology, and medical pharmacology and diagnostics.

Description of the Related Art

There is substantial evidence that somatic cells have a finite replicative capacity (Hayflick, 1965, Exp. Cell Res. 37: 614-636, and Hayflick, 1970, Exp. Geront. 5: 291-303) and that this process is a major etiological factor in aging and age-related disease (Goldstein, 1990, Science 249: 1129-1133; Stanulis-Praeger, 1987, Mech. Ageing Dev. 38: 1-48; and Walton, 1982, Mech. Ageing Dev. 19: 217-244). As cells undergo replicative senescence in vitro and in vivo, they not only lose the ability to divide in response to growth stimuli but also exhibit significant deleterious changes in the pattern of gene expression (West, 1994, Arch. Derm. 130: 87-95).

During replicative senescence, cells exhibit an elongation of the G_1 phase of the cell cycle, leading to a longer cell time of cycle transit. As the progression from mitotically active to senescent continues, cells fail to respond to mitotic signals and remain instead in G_1. The inability of senescent cells to enter the cell cycle represents a major difference between young and old cells in that young cells become quiescent entering G_0 until such time when they are induced to reenter the cell cycle and divide. Senescent cells exhibit changes in morphology, increasing in size and volume. However, senescent cells remain viable and are metabolically

active. Another characteristic of replicative senescence is that changes in the pattern of gene expression become more dramatic as the cell reaches the end of its replicative life. These changes are reflected in a decrease in the expression of "young-specific" genes with an increase in the expression of "old-specific" or "senescent-specific" genes. For purposes of the present invention, any gene whose product is differentially expressed between young quiescent cells and senescent cells is a "senescent-related" gene. Not only to do these changes affect the structure and function of the senescent cell, but such changes can also influence the physiology of surrounding cells and tissue matrix by altering the extracellular environment or in a paracrine fashion through the release of different proteins or through changes in cell-cell interactions.

Several senescent specific genes have been described in the scientific literature. Dermal aging is an illustrative example. Dermal aging is characterized by changes in the structure and function of extracellular matrix (ECM) proteins. Many of these same changes have been observed in experiments conducted with fibroblasts either grown to senescence or in cells derived from older individuals. Cells derived from older individuals exhibited up to a 4.4-fold greater level of fibronectin mRNA when compared to levels expressed in fetal cells. Similarly, the synthesis of fibronectin is increased in cells grown to senescence. In late passage cells, the fibronectin that is synthesized is structurally different from that observed in younger cells. These changes may reflect age-related changes in the processing of fibronectin mRNA. Functionally, these changes translate in a decreased capacity to mediate cell adhesion, cell spreading, and contact formation. When comparing younger and older cells, the fibronectin lattice appears different with the lattice in the older cells tending to

be less well organized. See Eleftheriou et al., 1991, *Cellular ageing related proteins secreted by human fibroblasts*, Mutat. Res. 256: 127-38; Kumazaki et al., 1993, *Enhanced expression of fibronectin during in vivo cellular aging of human vascular endothelial cells and skin fibroblasts*, Exp. Cell Res. 205: 396-402; Hara et al., 1993, *DNA-DNA subtractive cDNA cloning using oligo(dT)$_{30}$-Latex and PCR: identification of cellular genes which are overexpressed in senescent human diploid fibroblasts*, Analyt. Biochem. 214: 58-64; and Martin et al., 1990, *Fibronectin and collagen gene expression during in vitro ageing of pig skin fibroblasts*, Exp. Cell Res. 191: 8-13.

Another characteristic of dermal aging is that the expression of interstitial collagenase, also known as fibroblast collagenase, has been reported to increase in senescent cells as well as in cells derived from older donors. Not only is there an increase in the collagenase mRNA, but the activity of the enzyme is also increased. These effects appear to be at the transcriptional level and may in part be mediated by interleukin-1 (IL-1), which itself appears to be upregulated during senescence. See Sottile et al., 1989, *Regulation of collagenase and collagenase mRNA production in early- and late-passage human diploid fibroblasts*, J. Cell. Physiol. 138: 281-290; West et al., 1989, *Replicative senescence of human skin fibroblasts correlates with a loss of regulation and overexpression of collagenase activity*, Exp. Cell Res. 184: 138-147; Burke et al., 1994, *Altered transcriptional regulation of human interstitial collagenase in cultured skin fibroblasts from older donors*, Exp. Gerontology 29: 37-53; and Lafyatis et al., 1990, *Interleukin-1 stimulates and all-trans-retinoic acid inhibits*

collagenase gene expression through its 5' activator protein-1 binding site, Mol. Endo. 4: 973-980.

In addition, PAI-1 expression appears to be regulated as a function of dermal aging at both the mRNA and protein levels, although the mechanism is as yet unclear. See Shay et al., 1992, *Re-expression of senescent markers in deinduced reversibly immortalized cells*, Exp. Gerontology 27: 477-492. Stromelysin mRNA and protein are over-expressed in senescent cells (see Millis et al., 1992, *Metalloproteinases and TIMP-1 gene expression during replicative senescence*, Exp. Gerontology 27: 425-428; and Millis et al., 1992, *Differential expression of metalloproteinase and tissue inhibitor of metalloproteinase genes in aged human fibroblast*, Exp. Cell Res. 201: 373-379), as is tPA (see West, 1994, *The cellular and molecular biology of skin aging*, Arch. Dermatol. 130: 87-95). The levels of TIMP-2 protein and mRNA were studied in early and late passage human fibroblasts and found to be upregulated during senescence (see Zeng and Millis, 1994, *Expression of 72-kDa gelatinase and TIMP-2 in early and late passage human fibroblasts* , Exp. Cell Res. 213: 148-155).

Several other genes have been found to be over-expressed in senescent cells. Some of these genes appear to play a role in cell growth and signaling, and alteration in such genes may contribute significantly to an alteration in tissue physiology. IL-1 is upregulated during senescence, which can affect the transcription of several ECM genes, including stromelysin, PAI-2, and collagenase. See Kumar et al., 1993, *Expression of interleukin-1-alpha and ß in early passage fibroblasts from aging individuals*, Exp. Gerontology 28: 505-513; and Kumar et al., 1992., *Expression of interleukin 1-inducible genes and production of interleukin 1 by aging*

human fibroblasts, Proc. Natl. Acad. Sci. USA 89: 4683-7. IFN gamma can act to decrease the expression of several genes that are down-regulated during senescence. See Eleftheriou et al., 1991, supra; and Eleftheriou et al., 1993, *A group of three fibroblast secreted polypeptides suppressed by cellular ageing and interferon-gamma*, Biochim. Biophys. Acta 1180: 304-12. For example, IFN gamma functions to decrease the expression of collagen and to increase the expression of collagenase and fibronectin.

Fibroblasts are responsible for elastogenesis (see Braverman, 1989, *Elastic fiber and microvascular abnormalities in aging skin*, Clin. Geriat. Med. 5: 69-90), and an examination of the elastin produced by cultured dermal fibroblasts from individuals of increasing age reveals that, in the sixth decade, there is a marked reduction in the synthesis and repair of elastin fibers (see Fazio et al., 1988, *Isolation and characterization of human elastin cDNAs, and age-associated variation in elastin gene expression in cultured skin fibroblasts*, Lab. Invest. 58: 270-7; and Dalziel, 1991, *Aspects of cutaneous ageing*, Clin. Exp. Dermatol. 16: 315-23). Cultured dermal fibroblasts also exhibit an age-related decrease in collagen synthesis and an increase in degradation either when grown to senescence or when derived from people of various ages. See Mays et al., 1990, *Similar age-related alterations in collagen metabolism in rat tissues in vivo and fibroblasts in vitro*, Biochem. Soc. Trans. 18: 957; Furth, 1991, *The steady-state levels of type I collagen mRNA are reduced in senescent fibroblasts*, J. Gerontol. 46: B122-4; and Takeda et al., 1992, *Similar, but not identical, modulation of expression of extracellular*

matrix components during in vitro and in vivo aging of human skin fibroblasts, J. Cell. Physiol. 153: 450-9. Specifically, collagen type 1, pro alpha 1 and 3 chains, as well as type 3 pro alpha 1, are all down regulated during senescence at both the mRNA (see Hara et al., 1993, supra) and the protein level. See Dumas et al., 1994, *In vitro biosynthesis of Type 1 and III collagens by human dermal fibroblasts from donors of increasing age*, Mech. Age. Develop. 73: 179-187. Similar results were observed in experiments using cultured fibroblasts from pigs (see Martin et al., 1990, supra). In these studies, there was an increase in type III collagen, while type I collagen was synthesized but rapidly degraded. Thus, the ratio of type I:type III collagen was altered. These results demonstrate that age-related changes may be species-specific.

At least two proteins involved in the maintenance of the ECM are down-regulated in dermal aging: tissue inhibitor of metallo proteinase 1 (TIMP-1) is down-regulated (see West et al., 1989; and Millis et al., 1992, supra) as is osteonectin, a structural ECM protein induced during proliferation. See Reed et al., 1994, *TGF-beta 1 induces the expression of type I collagen and SPARC, and enhances contraction of collagen gels, by fibroblasts from young and aged donors*, J. Cell. Physiol. 158: 169-79. Early Passage Clone 1 (EPC1, see Pignolo et al., 1993, *Senescent WI-38 cells fail to express EPC-1, a gene induced in young cells upon entry into the G_0 state*, J. Biol. Chem. 268: 8949-8957), which is identical to Pigment Epithelium Derived Factor (PEDF, see Steele et al., 1993, *Pigment epithelium-derived factor: Neurotrophic activity and identification as a member of the serine protease inhibitor gene family*, Proc. Natl. Acad. Sci. USA 90: 1526-1530), is an example of a growth

factor that is down-regulated in senescent cells. Another gene that is down-regulated during senescence is a ribosomal protein, L7 (see Seshadri et al., 1993, *Identification of a transcript that is down-regulated in senescent human fibroblasts. Cloning, sequence analysis, and regulation of the human L7 ribosomal protein gene*, J. Biol. Chem. 268: 18474-80), while c-fos induction is also repressed during senescence. See Seshadri and Campisi, 1990, *Repression of c-fos transcription and an altered genetic program in senescent human fibroblasts*, Science 247: 205-209.

Thus, as an individual grows older, senescent cells make up an increasing percentage of the cells present in the tissues of the aging individual. The altered pattern of gene expression senescent cells exhibit contributes significantly to age-related pathologies. As the number of aged individuals is expected to increase dramatically in the near future, the cost of health-care for the aged will likewise increase dramatically. Reversal, partial reversal, or modulation of senescent gene expression can provide effective therapies for diseases, disease conditions, and pathologies in which replicative cell senescence is an etiological factor.

Consequently, there is a profound need for therapeutic agents and treatment regimes based upon the underlying biology of aging and age-related diseases, particularly the biology relating to the fundamental changes in gene expression that contribute to cell senescence and the development of age-related disease. The present invention helps meet that need by providing new methods for culturing senescent cells for use in cell-based assays and screens; for discriminating between genes expressed by young proliferative cells, young quiescent cells, and non-proliferating senescent cells; for separating young cells from senescent cells and using

those cells to conduct high-throughput screens based on cell senescence that can be used to identify compounds that, by reversing the senescent phenotype, treat or diminish age-related disease or pathologies; and for treating age-related human disease, as well as providing compounds and reagents useful in those methods.

Summary of the Invention

In a first aspect, the present invention provides a method for identifying and isolating senescence-related genes and gene products, which method comprises: (a) isolating mRNA from senescent cells and young quiescent cells; (b) amplifying said mRNA in a polymerase chain reaction to produce amplified gene sequences; (c) separating said amplified gene sequences by gel electrophoresis; and (d) analyzing said amplified gene sequences separated in step (c) to detect an amplified gene sequence from young quiescent and young dividing cells that is present at a level different from that observed in amplified gene sequences from senescent cells. With this method, one can readily identify and isolate senescence-related genes. For instance, the senescence-related genes or gene tags can be physically removed from the gel and sequenced, either directly or after cloning into a suitable recombinant DNA vector.

Thus, in a second aspect, the present invention provides useful nucleic acids in isolated form, which nucleic acids include portions of senescence-related genes and are useful as nucleic acid probes in diagnostic methods, as nucleic acid primers, and as components of recombinant DNA cloning and/or expression vectors. The present invention also encompasses the gene products of senescence-related genes.

In a third aspect, the present invention provides diagnostic methods for detecting senescent cells in culture and *in vivo* and for distinguishing senescent

cells from non-senescent cells. These methods comprise the steps of: (a) contacting the mRNA present in a cell or tissue with a labelled nucleic acid probe that comprises a sequence of a senescence-related gene under conditions such that complementary nucleic acids hybridize to one another; (b) determining whether specific hybridization has occurred; and (c) correlating the presence of senescent and non-senescent cells with the occurrence of hybridization.

These methods are especially useful in conjunction with therapeutic regimens and strategies. For example, one can use a probe (e.g., a nucleic acid or modified nucleic acid complementary to a senescence-related gene or RNA transcript or an antibody against a senescence-related gene product) to identify the senescent (or young) cells in a tissue sample. By appropriately labeling the probe for detection (e.g., with a fluorescent molecule or a molecule that serves as a binding partner for another molecule), one can label the senescent (or young) cells in a tissue and then separate the young from the senescent cells. In addition, a detectable reagent (e.g., a contrast molecule for magnetic resonance imaging) can be attached to an antibody or other substance which is specific for a senescence-related gene product, and can specifically label senescent cells. With such a preparation of cells enriched for either the young or senescent phenotype, one can then perform many useful procedures, including reintroduction of young cells into a host or treatment of senescent cells for reintroduction to the host.

In a related aspect, the identification of senescence-related genes can provide new therapeutic opportunities. Toxic substances (or "toxins") can be provided which are specific for senescent-specific (or senescence-related) gene products. Such toxins can kill cells expressing these senescent-specific or senescence-

related gene products. Toxins can be provided in the form of toxic substrates, wherein the toxic substrates can be activated (become toxic to cells) by senescence-related gene products. For example, beta-galactosidase is known to be a senescent-specific gene product, so one can prepare a toxic substrate (or "pre-toxin") that requires activation by beta-galactosidase to be activated and become toxic. Such a toxic substrate could be used to eliminate senescent cells *in vivo* or *in vitro*. In a further aspect, antibodies to senescence-related gene products can be linked with toxins or toxic substrates and delivered to cells by methods known to those in the art to destroy cells expressing senescence-related gene products. In this aspect, it is not necessary that the toxic substrates linked to the antibodies are activated by the senescence-related genes, as any type of activation known to those in the art (e.g., light, radiation, etc.) can be used. In another aspect, antisense oligonucleotides, can be targeted to senescence-related genes. Such antisense oligonucleotides can be comprised of ribonucleic acids, deoxyribonucleic acids, modified nucleic acids, or mixtures. Those of skill in the art will recognize that the senescence-related genes and gene products of the invention provide a wide array of such agents that can be used to target or direct therapeutic or diagnostic reagents to young or senescent cells.

In a fourth aspect, the present invention provides a method for screening compounds to identify compounds that can alter gene expression in senescent cells, which method comprises: (a) contacting senescent cells with a compound; (b) determining mRNA expression patterns in said senescent cells; and (c) correlating an alteration in mRNA expression of a senescence-related gene with a compound that can alter gene expression in senescent cells. The present invention also encompasses the

compounds identified by this method and the use of those compound to alter gene expression in senescent cells.

These and other aspects of the invention are described in more detail below.

Brief Description of the Drawings

The figures will first be briefly described.

Figure 1 shows three examples of enhanced differential display autoradiographs. The primer combinations are indicated above the gel. The lanes of the gel are as follows: (1) BJ cells old, Passage Doubling Level (PDL) 90.3, grown in 0.5% serum; (2) BJ cells young, PDL 40, grown in 0.5% serum; (3) IMR90 old, PDL 54, grown in 0.5% serum: (4) IMR90 young, PDL 21.4, grown in 0.5% serum; (5) IMR90 old, PDL 53, grown in 10% serum; and (6) IMR90 young, PDL 27.4 grown in 10% serum.

Figure 2 shows an analysis of the frequency of mismatches in the 3' most 8 bases of the 5' primers used in EDD: (a) frequency of mismatches detected in 34 genes; and, (b) distribution of the position of the mismatch in those sequences that have a single mismatch. Figure 3 shows differentially displayed bands in EDD analysis of young (Y) and senescent (O) fibroblasts. The loading order is identical to that of the gel shown in Figure 1. The arrows on the left indicate the differentially displayed band, with the numbers indicating the lane numbers with elevated expression. The sequence of the nucleic acids corresponding from bands comprising fragments of known genes are indicated on the right. The primer sets that were used for detection of the bands were 02 and C for Col3a1, 16 and C for Laminin A, 18 and C for ALDH-1 and 01 and D for IFN gamma.

Figure 4 shows Northern blots of RNA from young and senescent cells. 4A shows the loading order (for 4B, 4C

and 4D) of the RNA samples; 20 μg of total RNA were loaded in each lane, and loading was verified by ethidium bromide staining of an identical gel. The probes shown in 4B target genes previously reported (prior to September, 1995) to be expressed differentially between young and senescent cells. 4C shows probes for known genes that were identified by enhanced differential display to be expressed differentially; and 4D shows probes for novel genes that were identified by enhanced differential display to be expressed differentially.

Description of the Preferred Embodiments

In a first aspect, the present invention provides a method for identifying and isolating senescence-related genes and gene products, which method comprises: (a) isolating mRNA from senescent cells and young quiescent cells; (b) amplifying said mRNA in a polymerase chain reaction to produce amplified gene sequences; (c) separating said amplified gene sequences by gel electrophoresis; and (d) analyzing said amplified gene sequences separated in step (c) to detect an amplified gene sequence from young quiescent and young dividing cells that is present at a level different from that observed in amplified gene sequences from senescent cells. This method differs from prior art methods in that the method allows one to identify and isolate senescence-related genes rapidly and efficiently. A "senescence-related gene" refers to a gene that is expressed at a different level in a senescent cell than in a non-senescent cell of the same cell type. In some cases, a senescence-related gene will be expressed in a senescent cell and will either be expressed at a lower level or not be expressed at all in a non-senescent cell, in which case the gene is referred to as an "old-specific" gene. In other cases, a senescence-related gene will be expressed in a non-senescent cell and will

either be expressed at a lower level or not be expressed at all in a senescent cell, in which case the gene is referred to as a "young-specific" gene.

The advantages of this method in part result from a comparison of the mRNA populations of different populations, i.e., young quiescent cells and senescent cells. Of greatest interest and relevance is a comparison between young quiescent and senescent cells, because in vivo, most cells are generally in a quiescent state, unless there is need for proliferative activity, such as during wound-healing or tissue regeneration. A comparison between young quiescent cells and senescent cells therefore reflects the in vivo situation most accurately, and genes that are identified in this way have a high likelihood of being differentially expressed in the tissue. Those of skill in the art recognize that growth conditions can be modified to select for a population of cells that are mitotically active (typically, high serum concentrations and frequent passaging to keep the cells in a non-confluent state are used to keep the cells dividing, e.g., for fibroblasts, about 10% serum is adequate for this purpose) as opposed to quiescent (typically, low serum concentrations and contact inhibition, i.e., confluency, are used to keep the cells in a quiescent state, e.g., for fibroblasts, about 0.5% serum is adequate for this purpose). Typically, fetal or embryonic cells are not used unless one wants to detect developmentally-regulated genes.

The comparison of the mRNA population produced by each of these cell populations allows one to identify senescence-related genes. In other embodiments of the method, additional cell populations are used to provide additional information. Thus, while the method typically comprises comparing the mRNA population of young cells cultured in low serum (0.5%) with that of old cells cultured in low serum, one can enhance the method by

including in the comparison young cells cultured in high serum (10%) and old cells cultured in high serum. Young cells cultured in high serum with frequent passaging should be dividing and mitotically active, allowing one to detect mRNAs of growth-specific genes, such as those that encode proteins that regulate the cell cycle. Old cells cultured in high serum can produce mRNA species not seen in the mRNA populations of young cells cultured in high or low serum or old cells cultured in low serum. Furthermore, mere comparison of the mRNA population of young dividing cells with that of senescent cells might result in the identification of a gene product involved in cell cycling and cell division as a senescence-related gene product, because senescent cells do not divide. By using the mRNA population expressed by a young quiescent (non-dividing) cell for the comparison, one can avoid mistaking cell cycling and cell division gene products as senescence-related gene products.

The method is especially advantageous when used in conjunction with an mRNA preparation methodology known as "Enhanced Differential Display" or "EDD" and described more fully in U.S. patent application Serial No. 08/235,180, filed 29 April 1994, incorporated herein by reference. As the name implies, EDD is an improvement of methodology known as "Differential Display" or "DD". DD (see Liang, & Pardee, 1992, Science 257: 967-971) involves the use of PCR amplification of DNA fragments that represent the mRNA of a given cell population. One of the two primers used in the PCR is complementary to the poly-A tail of the mRNA, while the other primer (the 5' primer) has a randomly selected sequence intended to be complementary to an internal sequence within an mRNA. The annealing conditions for the 5'-primer, which is ten bases long, are chosen to be degenerate, so that only the last six to eight bases determine the sequence homology. Twelve different poly-T primers anchored by two

additional nucleotides are run in separate reactions in combination with defined but randomly selected 5'-primers.

Under these conditions, the assay will generate a display of about 30 to 50 to 100 bands that typically range in size from 100-400 base pairs (bp) per PCR reaction when resolved on a standard sequencing gel. The application of a sufficient number of primer combinations for each mRNA sample thus generates a catalog of bands (each band is called a "genetag") that represents the 3'-end of mRNA from expressed genes (or internal fragments of mRNA molecules that comprise an internal poly A tract). The displays of different mRNA populations can then be compared and differentially-displayed bands identified. These bands can then be cut out of the gel, sequenced and/or cloned, and the DNA fragment can then be used as a probe to isolate cDNA clones for a particular gene, as illustrated in the examples below.

Although DD has been used by many laboratories in its original form, the method has a tendency to exhibit extensive variability from experiment to experiment (see Liang & Pardee, 1993, Nuc. Acids Res. 21: 3269-3275; and Bauer et al., 1993, Nuc. Acids Res. 21: 4272-4280). This lack of reproducibility gives rise to many false positives, presumably due to the degeneracy of the PCR protocol (see Sun et al., 1994, Cancer Res. 54, 1139-1144). As a result, DD does not consistently allow the generation of a catalog of one mRNA population that can be compared to a catalog generated separately for another mRNA population. The improved process of the present invention, EDD, addresses the basis for the generally poor reproducibility of the DD technique.

The reliability of the DD technique appears to be in part dependent on primer-length and the temperatures at which primer extension is conducted. EDD essentially differs from DD in the use of longer primers and higher

temperatures for primer extension after a limited number of low temperature primer extension steps. By "longer primers" it is meant that the primers are greater than 18 nucleotides in length, and preferably are between 18 and 30 nucleotides in length. In DD, all of the primer extension steps are conducted at low temperatures. In the first few (2 to 6) primer extension steps in EDD, the temperature of primer annealing and extending is low, allowing primers to bind readily and not necessarily with complete complementarity to mRNA sequences. By "low temperature" or "low stringency" or "low fidelity" conditions, it is meant that the primer annealing/extending temperature is about 37°C to 50°C. Under these low stringency conditions, only the 3'-most (6 to 8) nucleotides of the primer dictate the specificity of hybridization. However, the primers used are much longer (20 to 30 or more nucleotides) than the hybridizing region active in the first few low-temperature cycles, and the later cycles of primer annealing and extending are conducted at higher temperatures that favor higher specificity and result in the replication of only primer extension products formed in the first few cycles, leading to more reproducible results. By "high temperature" or "high stringency" or "high fidelity" it is meant that the primer annealing/extending temperature is about 55°C to 75° C.

After a number of experiments with EDD, sufficient data was obtained to permit detailed analysis of primer annealing. One major question was the number of bases required at the 3' end of the arbitrary primer in determining specificity. From the described catalog (see below) and other experiments, 34 genes with known sequences were obtained. The site for the arbitrary primer annealing was determined by matching the gene sequences with primer sequences and band size. The complementarity for the eight bases at the 3' end were

determined. Matches with the gene sequences for bases 9-21 (3'-5') in the primer were highly variable and were not considered to contribute to the initial annealing of the primer. Figure 2 summarizes the mismatches in the 3' most 8 bases for 34 genes. Mismatches in the last eight bases were permitted and in some cases more than one mismatch was found. Mismatches occur more frequently toward the 5' end of the primer, as expected. Overall, the data in Figure 2 suggests that a 7 out of 8 match is the typical behavior of a 5' primer in EDD.

Using EDD, one can readily clone and sequence a 100 to 400 bp DNA fragment derived from the 3'-end of the mRNA from an expressed gene. Typically, one employs a total of 100 to 300 different primer combinations and generates from 1,000 to 10,000 (typically 4000-8000) different genetags, although one can get multiple genetags from the same mRNA. The genetags can be readily separated and visualized using gel electrophoresis or other techniques, and the genetags that are differentially expressed can be readily isolated, i.e., cut from the gel, and sequenced either directly or after cloning. Typically, about 50% of the sequences can be determined directly (from a gel isolated band), while the remaining bands must first be cloned to determine the sequence.

Each differentially expressed genetag (sometimes referred to as "band" due to the appearance of the genetag in a gel) isolated in this manner will typically first be used as a probe for Northern analysis to confirm the senescence-related nature of the genetag. RNA samples are prepared from young and old and mitotic and quiescent cells, and then probed with a labelled genetag probe to verify that the genetag is from an mRNA that is differentially expressed between young and senescent cells. Genetags that are indeed specific for young or old cells can then be used as probes for in situ RNA

analysis in tissue or organ sections of young and old donors, including both diseased and normal tissues or organs, to discriminate between young and old cells. Such analysis can be conveniently carried out using the reagents and in situ hybridization protocol described in the SureSite™ II System Manual, commercially available from Novagen. The genetags can also be used as probes for Northern analysis of RNA from cells of a type other than the cell-type from which the gene-tag was derived. Novel genes whose expression changes in association with aging can be cloned and further characterized using methodology well known in the art (see, e.g., Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Chapters 8 et seq. (2d ed., CSH Press, Cold Spring Harbor). For instance, the DNA sequence of novel genes can be analyzed by comparison to existing genes using standard molecular biology techniques and further analyzed to establish the function of the molecule encoded by the mRNA from which the genetag is derived. Antibodies can be raised against novel gene products to facilitate this analysis and to provide an antibody-based method for distinguishing young from old cells, or for other purposes, e.g., drug delivery, as described above. The gene products can also be incorporated in a senescent cell-based drug screen, as described more fully below.

The EDD methodology allows one to detect genes that exhibit a difference in the steady-state level of mRNA produced from those genes. Steady-state mRNA levels can be regulated at the transcriptional and post-transcriptional level. Old cells can also differ from young cells by altered steady-state mRNA levels and by altered levels of a protein or the activity of a protein, which can be due to alterations in mRNA translation or protein structure. Thus, regulation of gene expression can occur by a variety of mechanisms. At the

transcriptional level, the production of mRNAs can either increase or decrease. The level of translation or changes in post-translational modification can lead to an increase or decrease in the abundance of proteins. The activity of a protein can be modulated or the turnover rate of the protein can change. Each of these mechanisms can in turn be regulated. The methods of the present invention have resulted in the identification of a number of known and previously unreported genes and gene products, the expression or abundance of which is controlled at least in part by the aging process.

These gene products were identified in a study initiated to examine cell senescence in fibroblasts. Cultured dermal fibroblasts were selected for analysis in this study in part due to the extensive research conducted to date on cell senescence in this cell type (see, e.g., Harley et al., 1990, Nature 345: 458-460). Although cultured dermal fibroblasts were used, those of skill in the art will recognize that any cell type could be used in the methods of the invention. A non-exhaustive list of potentially useful cells are; (a) cells of the central nervous system, including astrocytes, endothelial cells, and fibroblasts, which are involved in such age-related diseases as Alzheimer's disease, Parkinson's disease, Huntington's disease, and stroke; (b) cells of the integument, including fibroblasts, sebaceous gland cells, melanocytes, keratinocytes, Langerhan's cells, and hair follicle cells, which are involved in age-related diseases of the integument, such as dermal atrophy, elastolysis and skin wrinkling, sebaceous gland hyperplasia, senile lentigo, graying of hair and hair loss, chronic skin ulcers, and age-related impairment of wound healing; (c) cells of the articular cartilage, such as chondrocytes, the senescence of which leads to the overexpression of the destructive proteins collagenase and stromelysin, which destroy

articular cartilage in osteoarthritis, and lacunal, synovial, connective tissue fibroblasts, which are involved in degenerative joint disease; (d) cells of the bone, such as osteoblasts, osteoclast progenitor cells, bone marrow stromal fibroblasts, and osteoprogenitor cells, which are involved in osteoporosis; (e) cells of the immune system such as B and T lymphocytes, monocytes, neutrophils, eosinophils, basophils, NK cells and their respective progenitors, which are involved in age-related immune system impairment; (f) cells of the vascular system, including endothelial cells, smooth muscle cells, and adventitial fibroblasts, which are involved in age-related diseases of the vascular system including atherosclerosis, calcification, thrombosis, and aneurysms; (g) cells of the eye, such as retinal pigmented epithelium, lens epithelial cells, iris muscle cells (myoblasts), and vascular endothelial cells, which are involved in loss of vision, i.e., age-related macular degeneration; (h) muscle satellite cells involved in muscular dystrophy; and (i) cells of the gut, such as intestinal epithelial cells, which are involved in malabsorption syndromes

The methods and reagents of the present invention in part arise out of the recognition that the structural and functional changes in organs and tissues that are intrinsic to the aging process can be attributed to an alteration in the pattern of gene expression that accompanies cell senescence. Research into cellular aging has provided insight into the mechanisms through which the lifespan of cells is regulated. The *in vitro* culture of normal diploid fibroblasts has served as a model system for studying cellular senescence and immortalization. Hayflick and Moorhead reported in 1961 that, with continuous passage, human diploid fibroblasts reach replicative senescence at a characteristic number of population doublings. Somatic cells derived from the

tissue of a young individual and grown in culture can divide a maximum of 50-100 times before reaching senescence. Furthermore, the upper limit in the number of cell divisions is inversely related to the age of the donor. Replicative senescence thus appears to be a genetically-programmed series of changes exhibited by normal cells that culminates in exit from the cell cycle and expression of a senescent phenotype.

As the body ages, the proportion of senescent cells within the skin increases. The accumulation of such cells is likely to have both direct and indirect effects that contribute to age-related changes and pathologies. As a cell becomes senescent, changes in the pattern of gene expression lead to functional changes. These changes can then influence the physiology of surrounding cells by altering the extracellular environment or in a paracrine fashion through the release of different proteins. For instance, the consequence of an accumulation of senescent cells within the skin is a progressive decrease in skin structure and function.

The present methods can be used in a number of applications to ameliorate the problems associated with the accumulation of senescent cells. For instance, the method can be used in the treatment of age-related lipofuscin accumulation in the retinas of humans and other animals. Lipofuscin accumulation correlates with macular degeneration. Recently, Eldred and Lasky (1993) characterized at least one of the components from the retinas of donors aged 52 to 98 years of age as N-retinylidene-N-retinylethanolamene, a Schiff base between retinaldehyde and ethanolamine. The source of the ethanolamine was proposed to be the rod outer segments that are rich in phosphatidylethanolamine and phagocytosed by the retinal pigmented epithelium (RPE). One theory for age related accumulation of lipofuscin is the decreased conversion of retinaldehyde to retinoic

acid in the aging RPE. The gene aldehyde dehydrogenase-1 (ALDH-1) is a senescence-related gene, as it is up-regulated in young quiescent cells. Because senescent cells show a phenotype of M1 activation, this gene cannot be induced in senescent cells. One of the activities of ALDH-1 is the conversion of retinaldehyde to retinoic acid. During cell senescence, the down-regulation of ALDH-1 can lead to increased retinaldehyde accumulation and therefore increased Schiff base formation with ethanolamine to form N-retinylidene-N-retinylethanolamine. The problem of decreased ALDH-1 in the formation of lipofuscin in aged RPE can be solved by gene therapy, i.e., by transfecting a recombinant constitutively expressed ALDH-1 into RPE cells to restore activity in aging cells and tissues, thereby decreasing the levels of lipofuscin. ALDH-1 activity could also be targeted in a screen for therapeutic agents that inhibit lipofuscin accumulation by increasing ALDH-1 activity. In addition, the gene for ALDH-1 could be added to aging cells by gene therapy .

A further use for the instant methods relates to treatment for AIDS. From recent studies, it appears that immune system dysfunction can result from the accumulation of senescent cells, as observed in AIDS patients and in the very old. There is evidence that senescent cells are refractory to apoptosis, and it has been reported that there is an increased number of lymphocytes in aged mice that are refractory to apoptosis. This correlates with decreased expression of FAS in the aged cells. When FAS is added transgenically, there is a marked restoration of normal immune function, even in old animals. It may be that the senescent cells that accumulate in aging humans and in AIDS patients are likewise refractory to apoptosis via a down-regulation of FAS levels or activity. If these long-lived, senescent lymphocytes have an inappropriate recognition of self, or

release growth inhibitory signals to prevent the proliferation of progenitor cells, then their presence could be deleterious. The instant method could be used to detect such senescent cells and treat the problems associated with their accumulation. For example, senescent cells can be eliminated via the administration of the FAS ligand or a similar molecule that is capable of activating the FAS receptor. This could stimulate apoptosis in these cells, clearing them where appropriate, allowing younger cells to replenish the circulating pool. The administration of such agents could decrease the percentage of senescent cells and decrease the phenomenon of autoimmunity or other immune system dysfunction. Molecules capable of activating the pathway via the receptor or other means could be discovered by placing senescent T lymphocytes in a cell-based screen for agents that induce apoptosis.

The methods of the instant invention also include the sorting of cells to distinguish senescent cells from young cells. Young cells (cells which have undergone fewer divisions) can be isolated from the tissue of donors, using a method of the instant invention, and prepared for use, i.e., reintroduction to the same donor. The cells with the greatest replicative capacity can be isolated and then grown in a culture medium which slows the replicative senescence of these cells. One method of slowing senescence is by the lengthening of telomeres, as discussed in "Methods and Reagents for Lengthening Telomeres," PCT/US94/13130, WO 95/13383, hereby incorporated by reference herein.

One cell type of particular relevance to the above described method is the melanocyte, a dendritic cell that inhabits the epidermis and produces melanosomes for inclusion in keratinocytes. The melanocyte is thought to replicate along with the basal keratinocytes and to possess a finite replicative capacity comparable to that

of other somatic cells. Senescent cells produce more melanin than the comparable young cell. The loss of melanocytes through replicative senescence may account for the hypopigmentation observed in aged skin and the poor tanning response, and the highly pigmented patches known as solar lentigines, or liver spots, may represent patches of senescent cells that overexpress melanin. Senescent melanocytes from a donor can be separated from young cells using the instant methods, cultured with an agent which slows or reverses replicative senescence, and reintroduced to the donor. Alternatively, one could administer a pre-toxin that is activated only in senescent cells.

These and many other therapeutic benefits can be realized with the present invention. The invention is illustrated by example below with respect to fibroblasts. To identify the senescence-related genes in fibroblasts, which may be responsible for or contribute to this decrease structure and function of aged skin, the cells used to illustrate the present method were BJ (foreskin) and IMR90 (lung) fibroblasts cultured in media containing either 0.5% or 10% serum. One could also employ fibroblasts derived from skin, such as fibroblasts isolated from fetal dorsal hand tissue. A series of 20 different 5'-primers and 12 different 3'-primers were used to amplify the mRNA, so 240 different primer sets were employed. The primers used in the amplification, together with their sequences and alpha-numeric designations, are shown in Table 1, below.

Table 1

Primers used in EDD for Human Fibroblasts

3'-(T-rich)-primers:

A:	5'-GCG CAA GCT TTT TTT TTT TTC T-3'	(SEQ ID NO. 1)
B:	5'-GCG CAA GCT TTT TTT TTT TTC C-3'	(SEQ ID NO. 2)
C:	5'-GCG CAA GCT TTT TTT TTT TTC G-3'	(SEQ ID NO. 3)
D:	5'-GCG CAA GCT TTT TTT TTT TTG T-3'	(SEQ ID NO. 4)
E:	5'-GCG CAA GCT TTT TTT TTT TTG G-3'	(SEQ ID NO. 5)
F:	5'-GCG CAA GCT TTT TTT TTT TTG A-3'	(SEQ ID NO. 6)
G:	5'-GCG CAA GCT TTT TTT TTT TTA T-3'	(SEQ ID NO. 7)
H:	5'-GCG CAA GCT TTT TTT TTT TTA C-3'	(SEQ ID NO. 8)
J:	5'-GCG CAA GCT TTT TTT TTT TTA G-3'	(SEQ ID NO. 9)
K:	5'-GCG CAA GCT TTT TTT TTT TTA A-3'	(SEQ ID NO. 10)
L:	5'-GCG CAA GCT TTT TTT TTT TTC A-3'	(SEQ ID NO. 11)
M:	5'-GCG CAA GCT TTT TTT TTT TTG C-3'	(SEQ ID NO. 12)

5'-(randomly-selected)-primers:

00:	5'-CGG GAA GCT TAT CGA CTC CAA G-3'	(SEQ ID NO. 13)
01:	5'-CGG GAA GCT TTA GCT AGC ATG G-3'	(SEQ ID NO. 14)
02:	5'-CGG GAA GCT TGC TAA GAC TAG C-3'	(SEQ ID NO. 15)
03:	5'-CGG GAA GCT TTG CAG TGT GTG A-3'	(SEQ ID NO. 16)
04:	5'-CGG GAA GCT TGT GAC CAT TGC A-3'	(SEQ ID NO. 17)
05:	5'-CGG GAA GCT TGT CTG CTA GGT A-3'	(SEQ ID NO. 18)
06:	5'-CGG GAA GCT TGC ATG GTA GTC T-3'	(SEQ ID NO. 19)
07:	5'-CGG GAA GCT TGT GTT GCA CCA T-3'	(SEQ ID NO. 20)
08:	5'-CGG GAA GCT TAG ACG CTA GTG T-3'	(SEQ ID NO. 21)
09:	5'-CGG GAA GCT TTA GCT AGC AGA C-3'	(SEQ ID NO. 22)
10:	5'-CGG GAA GCT TCA TGA TGC TAC C-3'	(SEQ ID NO. 23)
11:	5'-CGG GAA GCT TAC TCC ATG ACT C-3'	(SEQ ID NO. 24)
12:	5'-CGG GAA GCT TAT TAC AAC GAG G-3'	(SEQ ID NO. 25)
13:	5'-CGG GAA GCT TAT TGG ATT GGT C-3'	(SEQ ID NO. 26)
14:	5'-CGG GAA GCT TAT CTT TCT ACC C-3'	(SEQ ID NO. 27)
15:	5'-CGG GAA GCT TAT TTT TGG CTC C-3'	(SEQ ID NO. 28)
16:	5'-CGG GAA GCT TTA TCG ATA CAG G-3'	(SEQ ID NO. 29)
17:	5'-CGG GAA GCT TTA TGG TAA AGG G-3'	(SEQ ID NO. 30)
18:	5'-CGG GAA GCT TTA TCG GTC ATA G-3'	(SEQ ID NO. 31)
19:	5'-CGG GAA GCT TTA GGT ACT AAG G-3'	(SEQ ID NO. 32)

The amplification involved 4 cycles of degenerate (low temperature and fidelity) amplification, each cycle comprising 94°C for 45 sec.; 41°C for 60 sec.; and 72°C for 60 sec.; and 18 cycles of high temperature and fidelity amplification, each cycle comprising 94°C for 45 sec.; 60°C for 45 sec.; and 72°C for 120 sec. The amplified products were separated and visualized by polyacrylamide gel electrophoresis, and the differentially-displayed bands

were assigned a band number and then excised from the gel and either sequenced or cloned or both. An example of an EDD gel is shown in Figure 1 and examples of differentially displayed bands are shown in Figure 3. About 150 young and old-specific genetags of senescence-related genes were identified by this process. These genetags are summarized in Table 2, below.

The band number in Table 2 is a 4 digit number, the first two digits identify the 5'-primer used to generate the band, the third digit is a letter identifying the 3'-primer used to generate the band, and the fourth digit was assigned according to the number of differentially-displayed bands in a particular lane on the gel. The age number in Table 2 reflects the cells and media conditions in which the mRNA corresponding to the band is observed, according to the formula: O1 is BJ senescent cells, 0.5% serum; O2 is IMR90 senescent cells, 0.5% serum; O3 is IMR90 senescent cells, 10% serum; Y1 is BJ young cells, 0.5% serum; Y2 is IMR90 young cells, 0.5% serum; and Y3 is IMR90 young cells, 10% serum. If available, the Genbank locus designation is provided, and if the Genbank locus designation is not known, the term "Novel" is used to indicate that sequence information from the genetag is available but does not match any Genbank locus, and the term "Unknown" is used to indicate that sequence information is not yet available.

Table 2
Genetags Identified in EDD of Fibroblasts

Band No.	Age No.	Genbank Locus	Size (bp)
00C2	O1O2O3	HUMTIMPR	225
00D3	O1O2	Unknown	150
00E1	Y1	Unknown	135
00H1	O3	Unknown	185
00K1	Y1Y2Y3	HUMC1A2	450
00L1	O1	Unknown	450
00M2	Y1Y2	Novel	370
01C1	O1O2O3	HUMTPA	170
01C2	Y1Y2	Unknown	135

Table 2 (continued)
Genetags Identified in EDD of Fibroblasts

Band No.	Age No.	Genbank Locus	Size (bp)
01C3	O1	Unknown	235
01D1	O1	HUMSECP3	230
01E1	Y1	Novel	450
01E2	Y1Y2	Novel	320
01E4	Y1Y2	HSCOL3A1	137
01M4	O1(O2O3)	HUMINFGAMM	235
01M5	Y1	HUMBGR1A	145
01M6	Y1Y2	Unknown	130
02A1	Y1Y2(Y3?)	Unknown	>>500
02A2	O1O2(O3?)	MIT1HS	285
02B1	Y2	Novel	355
02B2	Y1Y2	Unknown	175
02C1	Y1Y2	HUMCG1PA1	200
02E3	O3	Unknown	148
02M4	Y1	Unknown	140
02M6	O2O3	Unknown	225
03C1	Y1Y2	Novel	>450
03C2	O1O2	Novel	380
03E1	O1O2	Unknown	>400
03F1	O2O3	Novel	>400
03F2	O2	Novel	200
03J1	O1	Unknown	250
03J1r	O1	Unknown	205
03J3	Y1Y2	Novel	240
03J3r	Y2	Unknown	190
03J4	O1O2	Unknown	330
03M1	O1O2(O3)	Unknown	245
03M2	Y1Y2	Unknown	190
03M3	O1O2O3	Novel	180
04D3	Y1Y2	Novel	200
04E2	Y2Y3	Unknown	180
04F2	Y1	unknown	175
04F3	O2O3	Unknown	160
04L2	O2O3	Unknown	170
04L3	Y1	Novel	135
04M1	Y1Y2	Unknown	240
05B1	Y2	Unknown	270
05C1	Y1Y2	Unknown	450
05C2	O1	Unknown	350
05C3	O1O2	Novel	280
05C4	O1	MnSOD	255
05D1	Y2	Novel	300
05D2	Y1Y2	Novel	260
05J1	O1O2(O3)	Unknown	160
05J2	Y1Y2	T08744	>500
05K1	Y1	Unknown	140
06D1	O1O2O3	Novel	215
06E1	O1O2O3	HUMTFPA	180
06E2	O1	Novel	150

Table 2 (cont.)

Genetags Identified in EDD of Fibroblasts

Band No.	Age No.	Genbank Locus	Size (bp)
06J1	O1	Novel	190
06L1	O1O2O3?	Unknown	>500
06L2	O1	Unknown	240
07C1	O3	Unknown	230
07C2	Y1Y2	Novel	190
07E1	O1O2O3Y3	T03598 IB568 (666)	140
07J1	O1	HUMIGFBP5	>500
07J2	Y1Y2Y3	Novel	180
07L1	O3	Novel	220
07L2	Y1	Unknown	220
07M1	(O1O2)O3	Novel	215
08B1	O2	Unknown	175
08D1	O1	Unknown	180
08D2	O1	Unknown	165
08D3	Y1Y2	Unknown	130
08D4	O3	Novel	115
08D5	O1	M78570	90
08E1	O1	Unknown	285
08E2	O1	Novel	230
08E3	O2O3	HUMSGP3	200
08F1	O1	M78570	295
08L2	O1O2O3	Unknown	150
08M1	Y1	Unknown	210
08M2	O2	Novel	130
09B1	Y1Y2	Unknown	230
09D1	O1O2O3	Novel	350
09D2	O1O2O3	Novel	120
09E1	Y1	T06399	155
09E2	Y2Y3	Novel	145
09J1	Y1Y2	Unknown	180
10C1	O1O2O3	Unknown	220
10D1	O1	HUMPAI2B	>500
10F1	(Y1)Y2	Novel	100
10J1	O1	Novel	190
10M1	(O1)O2	Unknown	240
10M2	Y1	Unknown	155
10M3	Y1Y2	Unknown	140
10M4	O1	Novel	115
11B1	O2	Novel	285
11E1	O3	HUMCILA	320
11E2	O3	Unknown	225
11E3	O1	Novel	150
11K1	Y1Y2	Unknown	190
11K2	O1O2O3?	Unknown	170
11M1	O2	Unknown	240
11M2	O1O2O3	Unknown	170
12F2	O2O3	HSCDN7	100
13C1	Y1	Unknown	125

Table 2 (cont.)

Genetags Identified in EDD of Fibroblasts

Band No.	Age No.	Genbank Locus	Size (bp)
13D1	O3	Unknown	185
13F1	O1	Unknown	130
13M1	Y1Y2	Novel	450
13M2	Y1Y2	Unknown	145
14F1	Y1	Unknown	130
14F2	O1O2	Unknown	110
14M1	O1	Novel	175
15M1	O2O3	Unknown	100
15M2	Y1	Unknown	90
16B1	O1O2O3	Unknown	230
16C1	Y1Y2	HSLAMA3	230
16C2	Y1Y2	T09243	90
16E1	Y2	Unknown	220
16F1	Y1	Novel	200
16F2	O1O2	HUMHERGC	160
16F3	O3	HUMCD44B	120
16F4	Y1Y2	Unknown	110
16H2	Y1Y2	Unknown	100
16J1	Y1Y2	Unknown	125
16K1	O1O2O3?	Unknown	280
16K2	O1	Unknown	190
16L1	Y2	Unknown	170
16M1	Y1	Unknown	250
17B1	O1(O2)O3	Unknown	330
17F1	O1	Unknown	320
17F2	O1(O2O3)	Unknown	200
17H1	O1	Unknown	260
17K1	Y1	Unknown	250
17M1	Y1	HUMSPARC	180
18C1	Y1	HUMALDHA1	330
18D1	O1	Unknown	290
18H1	O1	Novel	320
18H2	Y2	Unknown	220
18H3	Y2	Unknown	190
18J1	O1	Unknown	270
18M1	Y1	Unknown	330
18M2	Y2	Unknown	200
18M3	Y1	HUMKCS	140
18M4	O1	Unknown	110
19E1	O1	Unknown	330
19F1	O2O3	Unknown	280
19F1	O2O3	Unknown	280
19M1	Y1	Unknown	250
19M2	Y2Y3	Unknown	140

As demonstrated by Table 2, EDD of fibroblast cells resulted in the identification of many different

senescence-related genetags. Many of the genetags were from known genes, including both those known to be regulated with age and those not previously known to be regulated with age. For instance, IFN gamma has not previously been shown to be regulated with age. Band no. 00C2 corresponds to an mRNA with sequence homology to Genbank locus HUMTIMPR, which encodes TIMP (tissue inhibitor of metallo-proteinases) also known as erythroid-potentiating activity glycoprotein and collagenase inhibitor, and may encode TIMP 2, which has been reported to be present at higher levels in senescent cells (see Zeng & Millis, 1994, Exp. Cell Res. 213: 148). In addition, genetags specific for the mRNA of PAI2 and tPA, which have been previously reported to be old-specific gene products, and genetags for procollagen chains for type 1 and type 3 collagen, which have been previously reported to be young-specific gene products, were also identified by EDD.

A number of known genes were detected by several different primer sets, and it is expected that the same gene can be detected by different primer sets for the genes corresponding to the "Novel" and "Unknown" designations in Table 2. For instance, Genbank locus HUMTPA (encodes tissue plasminogen activator, also known as tPA) was identified using the primers sets defined by the following band numbers: 01C1, 01E3, 01F1, 02D1, and 03C3; Genbank locus HSCOL3A1 (encodes human pro-alpha 1 type 3 collagen) was identified using the primers sets defined by the following band numbers: 01E4, 01F2/3, 02C2/3/4, 02D2, 02E4, 02F3/4, 02H2, 02J1, 02K1, 02K2, 04C2, 04D2, 04L1, and 18K1 (and 01C2 may also be derived from this gene); and Genbank locus HUMCG1PA1 (encodes human pro-alpha I chain of type I procollagen) was identified using the primers sets defined by the following band numbers: 02C1 and 02E2.

Other known genes for which genetags were identified using EDD of fibroblasts include HUMC1A2 (encodes human pro-alpha 2 chain collagen type 1, band no. 00K1); HUMSECP3 (encodes human JE gene, which encodes a monocyte secretory protein, band no. 01D1, which comprises a sequence also in human interferon gamma, band no. 01M4); HUMBGR1A (encodes human glutamate receptor, band no. 01M5); MIT1HS (encodes mitochodrial RNA, band no. 02A2); HUMTFPA (encodes human tissue factor, band no. 06E1); HUMIGFBP5 (encodes human insulin-like growth factor binding protein 5, band no. 07J1; band no. 11H1 corresponds to Genbank locus HUMIGFBP5X); HUMSGP3 (encodes human secretory granule core proteoglycan, also known as (HSHPCP) hematopoietic proteoglycan and as (HUMSERG) a serglycin gene, band no. 08E3); HUMPAI2B (encodes human PAI-2, band no. 10D1); HUMCILA (encodes human lipoprotein-associated coagulation inhibitor; this genetag also comprises sequences of Genbank locus HUM0S14E01, from a human HepG2 3'- directed Mb01 cDNA, clone s14e01, band no. 11E1); HUMHERGC (encodes human heregulin beta 2 gene, a specific activator of p185-erbB2 (see Science 256: 1205 (1992), band no. 16F2); HUMCD44B (encodes human cell adhesion molecule CD44, band no. 16F3); HUMSPARC (encodes human osteonectin, also identified as aortic endothelial RNA, band no. 17M1); HUMALDHA1 (encodes human aldehyde dehydrogenase 1, band no. 18C1); HUMKCS (encodes human 80K-L protein, which is also known as calmodulin binding protein, protein kinase C substrate, band no. 18M3).

Other genetags could be correlated with known sequences in Genbank that have not yet been associated with a known gene or function. Thus, the present invention provides for the first time a utility for synthetic oligonucleotides comprising these gene sequences. For instance, band no. 05J2 corresponds to Genbank locus T08744 from Expressed Sequence Tag (EST)

06636, which shares sequence homology with EST02797 and EST00675, as well as to the GOS2 gene and/or alcohol dehydrogenase and human suilisol mRNA (HUMSUIISO); band no. 07E1 corresponds to Genbank locus T03598 IB568 (666) from a human cDNA clone known as IB568; band nos. 08D5 and 08F1 correspond to Genbank locus M78570 from EST00718, a cDNA clone homologous to tubulin alpha; band no. 09E1 corresponds to Genbank locus T06399 from EST04288, a human cDNA clone HFBDS91; band no. 12F2 corresponds to Genbank locus HSCDN7, a cDNA clone isolated using differential display as differentially expressed between androgen dependent and independent prostate carcinoma cell lines; and band nos. 16C2 and 16L2 correspond to Genbank locus T09243 from human sequence tag EST07136, a 3'-end clone HIBBR16. EST T09243 has now been identified in Genbank as human microfibril associated glycoprotein 4, HUMMFAPA, locus number L38486. The genetag 05C4, shown above in Table 2, has been identified as known gene Manganese Super Oxide Dismutase (MnSOD, see Kumar et al., 1993, Exp. Gerontol, 28: 505-13).

Other genetags identified using this method share homology with known genes. For instance, band no. 00D3 has sequence homology with the CD44 gene; band no. 00M2 has some homology with the human aromatase cytochrome P-450 gene; band no. 03M3 has a rich G(1-2)A(1-3) stretch and so shares sequence homology with the human pepsinogen gene, the PAI1 gene, and some human sequence tags (the GA region seems to be a repeating motif in some genes); and band no. 16C1 share sequence homology with the human laminin A gene.

With respect to the genetags identified in Table 2 as "Unknown", these genetags can be cloned and then used as a probe for Northern analysis of RNA samples of young and old, mitotic and quiescent fibroblast cells, to verify that the genetags are differentially expressed. Once the

verification is made, the genetags can be sequenced and then identified as either a known or previously unknown gene. Once identified, senescence-related genes, probes specific for those genes, the gene products of those genes, and antibodies to those gene products can be used as markers for detecting senescent cells, for distinguishing between and/or separating young and old cells, and for screening and/or therapeutic purposes. For screening purposes, the gene product of a senescent gene will be a useful target for therapeutic intervention if that gene product is involved in disease pathology or if a change in its expression parallels that of gene products involved in disease pathology. One can quantitate changes in the level of gene expression caused by a compound using high-throughput screening techniques. Using active compounds, one can determine whether and at what level coordinate modulation of gene expression occurs (i.e., does the compound affect senescence-related genes globally, in groups, or individually), and if by group, to which group an individual gene belongs. The set of genes initially chosen for use in such screening can be modified as screening results accumulate.

Table 3 below summarizes the data collected from the EDD performed on fibroblast cells.

Table 3
Summary of EDD Results on Fibroblasts

Cells	Known Genes		Novel Genetag	EDD Bands	Total
Young IMR90	0		3	9	12
Young IMR90/BJ	6	collagen 1, pro a 2 collagen 3, pro a 1 (14x) collagen 1, pro a 1 (2x) laminin A EST06636 EST07136 (2x)	10	16	32
Young BJ	5	aldehyde dehydrogenase glutamate receptor 80K-L protein osteonectin EST04288	2	13	20
Old IMR90	5	lipoprotein-associated coagulation inhib. hematopoetic proteoglycan CD44 HSCDN7 tPA (3x)	6	13	24
Old IMR90/BJ	1 5	tPA (2x) TIMP 2 human tissue factor (EST) IB568 heregulin mitochondrial RNA	7	15	28
Old BJ	6	IGF binding protein 5 PAI-2 (uPAI) MnSOD interferon gamma (2x) EST00718 (2x)	9	18	32
Total	27		37	84	148

Most of the genes and gene products noted in Tables 2 and 3, above, have not previously been identified as products of senescence-related genes. Many of the gene products are secreted proteins, which is consistent with the alteration in extracellular matrix observed in aging tissues. Thus, the present invention provides novel methods and reagents for identifying senescent cells in tissue or culture, which methods generally comprise determining whether a cell expresses a senescence-related gene product, which can include an mRNA or other RNA or a

protein, and correlating the presence of that gene product with the state of senescence of the cell or tissue.

Typically, such methods will be practiced using oligonucleotide probe hybridization to the mRNA of the cell, either in situ or in a cell extract. In one such method, probes specific for the mRNA corresponding to a senescence-related gene are immobilized on a membrane or filter. Then, the cells of interest are cultured under conditions conducive to gene expression and flash-frozen. The cells are then thawed in the presence of a labelled mRNA precursor, so that the label is incorporated into transcripts that were being transcribed when the cells were frozen. The labelled mRNA is then harvested from the cell and hybridized to the immobilized probes on the filter. The pattern of hybridization will identify whether senescence-related genes are being expressed by the cell. Those of skill in the art readily understand how to make probes specific for a particular gene product provided the sequence of the gene or mRNA produced by the gene is known.

Consequently, although the sequence of known genes is not repeated herein, but the sequence of genetags corresponding to novel senescence-related genes is provided in Table 4, below. The sequences are identical to the RNA identified by EDD but for the substitution of deoxyribonucleotides for ribonucleotides and are identified by band no. and shown in the 5'-to-3' direction (N is any base and indicates that the identity of the nucleotide at that position is not known).

Table 4
Novel Sequences from EDD of Fibroblasts

00M2
CATTTATTCA TTCATTGAGA CACTCAA (SEQ ID NO. 33)

01E1
ACAGAAAGGC CACTCAGGAT GTCCTTTGTG TCCATTGATG TCATTCAGCA GTCAGTCCCC CAATAATCCT TAAACTAGCT AAAACCAAAG GTAGTCNTTA GAAGATCTGC TT (SEQ ID NO. 34)

01E2
TTGAGTAGTT ACTGGAACCT TGACATTGCC TTTTAATGAG GTACTTCCAA AAAAAGGACC CCTAACAATG GCATAATAGT GAGGTCTCTC TGTGCGTGTA CATAATATA (SEQ ID NO. 35)

02B1
CAAAGATAAG AAACCAAGGA AGAAAGCAA (SEQ ID NO. 36)

03C1
CTGACGCCAN CCGCATACNC CGCANCCACA (SEQ ID NO. 37)

03C2
AGATAAAGCA ATTAGAAGAT GCATTAAAAG ATGTGCAGAA GAGGATGTAT GAGTCAGAAG (SEQ ID NO. 38)

03F1
ATAATAAAAC TCTTCATTTT GCGAATTATA GAAGCTACTT TTTATAAAGC CATATTTTTT TAGGGAAACT AAGGAGTGAC ATAGAA (SEQ ID NO. 39)

03F2
AACTGCATTT TGATGTTATC GCTTATGTTT AATAGTTAAT TCC (SEQ ID NO. 40)

03J3
CTATTGCCTC TCCTCCTGCA GAGACCATG (SEQ ID NO. 41)

03M3
GAGAAGAAAG GAAAGAAAGG NCACAGAGAT GGAAGGCCA (SEQ ID NO. 42)

04D3
GTTTCTGAAT TACATGAATT GTTGCAGAGC AAAGAAACTT ATGGAAATCT TTCCATTTAT (SEQ ID NO. 43)

04L3
GTAGGCTTCT ATATTGCATT TAACTTG (SEQ ID NO. 44)

05C3
AATGAGGTAG AAGTAGAAAG GAAGAAAAAC TCAAAGAATT CTAAAAGGAT TCATAGCAAC ATAATGTGTC CC (SEQ ID NO. 45)

Table 4 (cont.)
Novel Sequences from EDD of Fibroblasts

05C4
TCTCACATTC AGTCATACCC TAATGATCCC AGAAAGATAA TCAT
(SEQ ID NO. 46)

05D1
AGAAGCCCCA GCAAGATTTA TTCCTTTTTG CTTCTTCATA ACCATGAAGC
CATTGAAC (SEQ ID NO. 47)

05D2
CTACCTCCCA CATTAATTTT CATATGT (SEQ ID NO. 48)

06D1
AGGGCACAGC ACCAGATGAA TTGTTGTATA T (SEQ ID NO. 49)

06E2
AAATTAGCTT TCATCACAGA TTTAGGAAACT TGTCT
(SEQ ID NO. 50)

06J1
AAACTACTGA ACNGTTACCT AGGTTAACAAC CCTGGTTGAG TATTTGC
(SEQ ID NO. 51)

07C2
TTGNATATTG NATTTGTAGT AATATTCCAAA AGAATGTAAA TAGG
(SEQ ID NO. 52)

07J2
AAATTGTATA TTGTATTTGT AGTAATATTCC AAAAGAATGT
(SEQ ID NO. 53)

07L1
TATGAATNTC ACATTTGAAT TCTTCGATCTC TAA
(SEQ ID NO. 54)

07M1
TATGTATAAA AGCATATGTG CTACTCATCTT TGCTCAC
(SEQ ID NO. 55)

08D4
AATGTCTAAT TTTCTTTCCG ACACATTTACC AAA
(SEQ ID NO. 56)

08E2
ACAACAGCAA ACAAAAAGGT GAAGTCTAAAT GAAGTGCACA
(SEQ ID NO. 57)

08M2
AAAAGAATTG GCAGTTACAT TCATACTTT (SEQ ID NO. 58)

Table 4 (cont.)

Novel Sequences from EDD of Fibroblasts

09D1
AAGAATGTGC ATTCCAGTGC CATAGATAGT ATATTGAA
(SEQ ID NO. 59)

09D2
TTGCTACGGA CTTACGAAAG GACAAAGCGA AGAGCTG
(SEQ ID NO. 60)

09E2
AAATAATTTA TTCATTGCAG ATACTTTTTA GGTTGCATTT
TATTCATTTC C (SEQ ID NO. 61)

10F1
AGATGATGAT GTTAACCCAT TCCAGTACAG TATTCTTTT
(SEQ ID NO. 62)

10J1
AGTATAGTGA ATGANTATGC CTTCCTACTG CAG (SEQ ID NO. 63)

10M4
AGAAATATAA AGATTTTNAT ACCTGCCACA TGG (SEQ ID NO. 64)

11B1
GAAGANATTA TGTTGTGANC NGGAGTNACA CAAA (SEQ ID NO. 65)

11E3
AGGGGCACAA GAGTTTGCGG TTATTGAATC CTGAGANAA
(SEQ ID NO. 66)

13M1
GTTGAAGAGA CAGAGACAAG TAATTTGC (SEQ ID NO. 67)

14M1
CCGTGAATAC CCNTTTCTCG ACCAAAGA (SEQ ID NO. 68)

16F1
ATGGAGTTGT GGATGAAAGC CATGTTAGNT G (SEQ ID NO. 69)

18H1
GATCATATAA ACANNNCCGA GTTCTACCTC AGAGTCG
(SEQ ID NO. 70)

Those of skill in the art will recognize that the complete coding sequence of a gene corresponding to a genetag of the invention, as well as the endogenous promoter and other regulatory elements of the gene, can be readily isolated once the sequence of the genetag or

the genetag itself (which can be generated using the primers indicated above) is known or available, as provided by the present invention. Such genes can be used, either directly or after suitable modification using standard techniques of molecular biology, not only to express the mRNA or protein encoded by the gene but also to express antisense oligonucleotides or ribozymes that can be used to prevent deleterious expression of senescence-related genes. Those of skill in the art recognize that a wide variety of expression plasmids can be used to produce useful nucleic acids of the invention and that the term "plasmid", as used herein, refers to any type of nucleic acid (from a phage, virus, chromosome, etc.) that can be used to carry specific genetic information into a host cell.

To verify that the genetags are differentially expressed, probes for known and novel genes were prepared and tested on Northern blots. Northern and Southern blot analysis was performed according to standard procedures (Sambrook, J., Fritsch, E.F., & Maniatis, T. (1989) Molecular Cloning; a laboratory manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor). Nucleic acids were transferred to charged nylon membranes, then cross-linked using a UV STRATALINKER (Stratagene). Probes for novel genetags were prepared by restriction digestion of the appropriate plasmid, which contains the novel genetag cloned into it, with *Hind*III followed by isolation of the insert band on low-melting agarose and then radioactive labeling of the DNA using the random hexamer-primed method (Feinberg & Vogelstein, 1983, Anal. Biochem. 132:6-13). Following hybridization to the probe, the filter blot was washed twice in 1x SSC and 0.5% SDS at 65°C for 30 minutes. The blots were then exposed and analyzed using a PhosphorImager 425E (Molecular Dynamics). Probes for known genes were prepared by kinase end labeling of approximately 40-mer

oligos that were designed to be complementary to the 5' coding region of the message. The following probes for known genes were used:

80K-L protein, Human, GenBank Accesion Number (GBAN): D10522, bases: 497-458, 5'-CCG TTT ACC TTC ACG TGG CCA TTC TCC TGT CCG TTC GCT T-3' (SEQ ID NO.71);

Aldehyde Dehydrogenase 1, Human, GBAN: K03000, bases: 186-147, 5'-AGG AAC AAT ATT CAC TAC TCC AGG AGG AAA CCC TGC CTC T-3' (SEQ ID NO. 72);

Cell Adhesion Molecule (CD44), Human, GBAN: M59040, bases: 166-127, 5'-CCG AGA GAT GCT GTA GCG ACC ATT TTT CTC CAC GTG GTA T-3' (SEQ ID NO. 73);

Collagenase, Human, GBAN: X05231, bases: 181-142, 5'-CCA GGT ATT TCT GGA CTA AGT CCA CAT CTT GCT CTT GTG T-3 (SEQ ID NO. 74)';

Collagen 1 alpha 1, Human, GBAN: K01228, bases: 990-951, 5'-ATC AGC ACC TTT GGG ACC AGC ATC ACC TCT GTC ACC CTT A-3' (SEQ ID NO. 75);

Collagen 1 alpha 2, Human, GBAN: J03464, bases:519-481, 5'-AAG GTT ACT GCA AGC AGC AAC AAA GTC CGC GTA TCC ACA A-3' (SEQ ID NO. 76);

Collagen 3 alpha 1, Human, GBAN: X14420, bases: 142-103, 5'-CGA GAA GTA GCC AGC TCC CCT TTT GCA CAA AGC TCA TCA T-3' (SEQ ID NO. 77);

Elastin, Human, GBAN: M36860, bases: 183-144, 5'-CCT GGA TAA AAG ACT CCT CCA GGA ACT CCA CCA GGA ATG G-3' (SEQ ID NO. 78);

EPC-1/PEDF, GBAN: M76979, bases: 256-217, 5'-AAG AAA GGA TCC TCC TCC TCC ACC AGC GCC CCT GTG CTG T-3' (SEQ ID NO. 79);

Heregulin-beta 2, Human, GBAN: M94167, bases: 654-615, 5'-GAG GAG TAT TCA GAA CTG GTT TCA CAC CGA AGG ACT AGT T-3' (SEQ ID NO. 80);

Human Tissue Factor, GBAN: M16553, bases: 357-318, 5'-CTC GTC GGT GAG GTC ACA CTC TGT GTC TGT TGT GTA AAA C-3' (SEQ ID NO. 81);

IGF Binding Protein 5, Human, GBAN: M62403, bases: 538-499, 5'-CTG GTG CTC CGG TCT CGA ATT TTG GCG AAG TGC TTC TGC A-3' (SEQ ID NO. 82);

Laminin A, Human, GBAN: X70904, bases: 193-154, 5'-CTC CAT ATT GAT AGG CGT GCT CTA TTG CTC TAG GGC TGT T-3' (SEQ ID NO. 83);

PAI-1, Human, GBAN: M16006, bases: 340-301, 5'-TCT TGA ATC CCA TAG CTG CTT GAA TCT GCT GCT GGG TTT C-3' (SEQ ID NO. 84);

PAI-2, Human, GBAN: M18082, bases: 74-34, 5'-ATT GAG GGC AAA GAG TGT GTT TGC CAC ACA AAG ATC CTC C-3' (SEQ ID NO. 85);

TPA, Human, GBAN: X13097, bases: 299-259, 5'-TTG CTT CTG AGC ACA GGG CGC AGC CAT GAC TGA TGT TGC TG-3' (SEQ ID NO. 86); and,

UPA, Human, GBAN: K03226, bases: 405-366, 5'-ATC TGT GGG CAT GGT ACG TTT GCT GAA GGA CAG TGG CAG A-3' (SEQ ID NO. 87).

Genetags that could be readily sequenced were used for further analysis of the data generated by EDD. Oligonucleotide probes for Northern analysis were designed that were complementary to many of the known genes listed in Table 3 (see above). Five probes were prepared for genes that were previously characterized as being differentially regulated in young and senescent cells (PAI-1 (Goldstein, et al., 1994, J. Cell Physiol. 161: 571-9), elastin (Fazio, M.J., et al., 1988, Lab. Invest. 58: 270-7.), EPC-1 (Pignolo, et al., 1993, J. Biol. Chem. 268: 8949-8957), collagenase (West, et al., 1989, Exp. Cell Res. 184: 138-147.) and urokinase type plasminogen activator (Shay, et al., 1992, Exp. Gerontology 27: 477-492.); see Figure 4B). RNA was prepared from both young and senescent IMR90 and BJ fibroblasts grown in either 0.5% or 10% FBS. These 8 RNA samples allow a comprehensive analysis of the expression

of genetags identified by EDD. Northern analysis of previously characterized genes with altered expression in young or senescent cells was performed for all 5 genes and the reported differential expression was confirmed (see Figure 4B). For reasons of convenience, the 8 RNA samples were loaded for Northern analysis in the order shown in Figure 4A. Of the 21 known genes identified by EDD (not including ESTs), Northern analysis confirmed differential expression for 12 genetags (see Figure 4C), including the four differentially displayed bands from Figure 3, while 7 genetags failed to show the predicted differences (data not shown). The hybridization pattern of one probe was non-specific, and one gene (mitochodrial RNA) was not analyzed in this analyis.

During the cloning of novel genetags, it was observed that a band that generated an identifiable sequence sometimes gave rise to plasmids with different inserts. In such cases, several clones for each band were isolated and a clone that contained the initial sequence was used to probe Northern blots. Of the 37 novel genetags (Table 3), 31 were recovered after cloning. Two genetags were identified twice in EDD, leaving 29 unique genetags to be tested. The conditions for specific hybridization of probes for novel genetags were first tested in Southern blot analysis. Genetags are most often generated from the untranslated 3' end of the message, a region rich in repetitive-type sequence. Of the 28 probes that detected specific bands in Southern analysis, 5 probes did not give a signal in Northern analysis (20 µg of total RNA was loaded per lane), 12 probes did not confirm the EDD observations, and 11 probes (see Figure 4D) were in agreement with the initial EDD observation. This analysis again demonstrates that about half of the genetags tested confirmed the initial EDD observation.

Novel genes which were shown to be differentially expressed using the instant methods are shown in Table 5 below, which also shows the approximate size of mRNA detected in Northern blots as well as the size of insert cloned into the genetag cloning vectors. The reference numbers for each genetag are also shown.

Table 5

Geron Reference	Approximate mRNA Size	cDNA Insert Cloned	Note
09D2	2.9 kb	2.9 kb	identical to 08E2
05C4	2.9 kb	2.4 kb	identical to MnSOD2
08E2	2.9 kb	2.9 kb	identical to 09D2
10J1	5.3;3.6 kb	3.9 kb	
10M4	3.9 kb	NA	
11E3	3.3;2.7 kb	2.5 kb	
18H1	6.0;3.8 kb	4.3 kb	
03J3	14;11;3.8 kb	3.8 kb	
04L3	3.7 kb	3.5 kb	
16F1	3.7 kb	2.0 kb	
07L1	3.5 kb	2.0 kb	identical to 08D4
10F1	5.4;3.6 kb	5.4 kb	
05D1		6.5 kb	

Those in the art will recognize that the given mRNA sizes in the above table are only approximations. Figure 4D shows examples of Northern blots of mRNA similar to blots from which the above listed mRNA sizes were obtained.

Sequence data available for these senescence-related novel genes is given below in Table 6; the sequences are also identified by Geron reference number. The term "contig" identifies a sequence of a genetag for which another noncontiguous sequence is also available. The

sequences are shown in the 5'-3' direction. N is any base and indicates that the identity of the nucleotide at that position is not known. M indicates that the nucleotide at that position is either A or C. R indicates that the nucleotide at that position is either A or G. W indicates that the nucleotide at that position is A or T/U. Y indicates that the nucleotide at that position is C or T/U. K indicates that the nucleotide at that position is G or T/U.

Table 6

Senescence-Related Novel Gene Sequences

08E2, AT7

GCGGCGGCCA TGGCGGGACA GGAGGATCCG GTGCAGCGGG
AGATTCACCA GGACTGGGCT AACCGGGAGT ACATTGAGAT
AATCACCAGC AGCATCAAGA AAATCGCAGA CTTTCTCAAC
TCGTTCGATA TGTCTTGTCG TTCAAGACTT GCAACACTAA
ACGAGAAATT GACAGCCCTT GAACGGAGAA TAGAGTACAT
TGAAGCTCGG GTGAC (SEQ ID NO. 88)

08E2, contig 1

CCAGCAATCT ATCATGGATC CTAATCAGAA CGTGAAATGC
AAGATAGTTG TGGTGGGAGA CAGTCAGTGT GGAAAAACTG
CGCTGCTCCA TGTCTTCGCC AAGGACTGCT TCCCCGAGAA
TTACGTTCCT ACAGTGTTTG AGAATTACAC GGCCAGTTTN
GAAATCGACA CACAAAGAAT AGAGTTGAGC CTGTGGGACA
CTTCGGGTTC TCCTTACTAT GACAATGTCC GCCCCCTCTC
TTACCCTGAT TCGGATGCTG TGCTGATTTG CTTTGACATC
AGTAGACCAG AGACCCTGGA CAGTGTCCTC AAAAAGTGGA
AAGGTGAAAT CCAGGAATTT TGTCCCAAAT ACCAAAATGC
TCTTGGTCGG CTGCAAGTCT GATCTGCGGA CAGATGTTAG
TACATTAGTA GAGCTCTCCA ATCACAGGCA GACGCCAGTG
TCCTATGACC AGGGGGCAAA TATGGCCAAA CAGATTGGAG
CAGCTACTTA TATCGAATGC TCAGCTTTAC AGTCGGAAAA
TAGCGTCAGA GACATTTTTC ACGTTGCCAC CTTGGCATGT
GTAAATAAGA CAAATAAAAA CGTTAAGCGG AACAAATCAC
AGAGAGCCAC AAAGCGGATT TCACACATGC CTAGCAGACC
AGAACTCTCG GCRGTTGCTA CGGACTTACG AAAGGACAAA
GCGAAGAGCT GCACTGTGAT GTGAATCTTT CATTATCTTT
AATGAAGACA AAGGAATCTA GTGTAAAAAA CAACAGCAAA
CAAAAAGGTG AAGTCTAAAT GAAGTGCACA GCCAAAGTCA
TGTATACCAG AGGCTTAGGA GGCGTTTGAG AGGRTACTCA
TCTTTTTGGG AATCCTGACC TTAGGTTCGG CATGTAGACC
AAGTGATGAG AAGTGAATAC ATGGAAGAGT TTTTAAGTGT
GACTTGAAAA ATATGCCAAA AAATGAGAGA TACAAATGAG
CTAGAGGAAG ATGAGGGGGG ATGCGAGTAC CTCCAAGAAG

AAAAATCACA CTCTGAATGG TGCTTGCATT TTGAGGTTTN
NNNNCNNNNG GGGTATAATC TATCATGGAT CTCCACTTGG
ATTAATTTTA AATGTTTAAT CTCCTTTACA AAAAGTATAC
GTTAATATAC CGTCCTCAAG GGGGACTGGC ACTGTGACCT
AGCATTAGTT TCTAGAGGAT GTGATCTAAT TCTTCTAGCT
CATCATAAAA AGGAATTGTA TCAGGACCCA TGGGATATAT
CCAGAGGCAA CTTATGAGGC TTGGAATCTG GCTTCCTGAA
GATAGCTGAG TAGGATGGTC TAAGGAAAGC CTTGGAATCT
TGCAAGATTG GTGGACCAGC ACTACAAAGA TCGCATAGAT
CAAATAGGAA AAAAATGTCG ATTTTTATTC AGTCTGATGG
TTCTGTTCTC ATGGTGGATG GTCATAAAAA GTGG
(SEQ ID NO. 89)

08E2, contig 2
GGCACGAGCG GAATTGGACT TGGGAGGCGC GGTGAGGAGT
CAGGCTTAAA ACTTGTTGGA GGGGAGTAAC CAGCCTGCTC
CTCTCGCTCT CCTCCTCGTC TGCGCCGCGT TTCAGAGGTT
GCCCATCAGC CTTGTGATTT ATTTTTATAT CTGCTTTTTA
TAAAGAGAGA AATATATATA TATATATATA TATTTTTTTT
TTCTTCTTAA GAGAAAATTC CTGTTCCAAG AGAAAATAAG
GCAACATCAA TGAAGGAGAG AAGAG (SEQ ID NO. 90)

08E2, contig 3
GATTGATGCA GCATTATGCT TTGGGCAGTA TTACAAAATA
GCTGGCGAGT KCTTTCTGTA TTTAAATATT GTAAAAAGAA
AATAAGTTAT AACTGTTATA AAGCAGAACT TTTGTTGCAT
TTTTTAAACT GTTGAAGTCA CTGTGTATGT TTGTTTGGTC
AATGTTTCCG CAGTATTTAT TAAAACATAC TTTTTTTTTT
CTTCAAATAA AAAAGTAACC ATG (SEQ ID NO. 91)

08E2, DT7
TCCTCTCGCT CTCCTCCTCG TCTGCGCCGC TTTCAGAGAG
AAAATTCCTG TTCCAAGAGA AAATA (SEQ ID NO 92)

10J1
ACCATGGAAA GTATAGTGAA TGAATATGCC TTCCTACTGC
AGCAAAACTC AAAAAAGCCC ATGACAAATG AGAAACAAAA
TTCCATTTTG GCCAACATTA TTCTGAGTTG TCTAAAGCCC
AACTCCAAGT TAATTCAACC ACTTACCACG (SEQ ID NO. 93)

10J1, 3T3
GTTTTTTTTG AGTTTAACAC AGATTTTATT GCCCTATAGA
CAGTTATGAT GTGACCAGTG GATATCAATG AAACTTCTTA
ATTATTTGAG TCTGAAAATG CATATTTA (SEQ ID NO. 94)

10J1, 3T7
ACAAACCACA GTATTTCCAC TTTAAATATA GAACTGGTAA
ACAGCACTAT CCTTAAACTA AAATCGGTGA CTCGGTCATC
AAGAAGGTTT TTGCCCGCCC GTGGATC (SEQ ID NO. 95)

10J1, 4T3

TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT GAAAGAATAG
GTTTAATTTA TTAGTTGCTC TTTAGCAAAG GCTATATAGA ACA
(SEQ ID NO. 96)

10J1, 4T7
GCTAAACCAA ACCAACTCCT CTGCTTTGTC CCTTAGGTGG
AAAAGAGAGG TAGTTTAGAA CTCTCTGCAT AGGGGTGGGA ATTAAT
(SEQ ID NO. 97)

10M4
TGTTATTATT AGCTTTCCAT GTGGCAGGTA TTAAAATCTT
TATATTTCTG AAAAATACTC TTCTCTT (SEQ ID NO. 98)

11E3
GATGGGGACG TCCTGATTTA CCAGATCAAA GTATGGTAAG
GCTGTAGATA GCACACTAGT TTTCTCAGGA TTCAATAACC
GCAAACTCTT GGTGCCCCTA TTG (SEQ ID NO. 99)

11E3, 4T3
TTTTTTTTTT TTTTTTTTTT TTAGGAAAGA AAGAATTCTT
TTATTCCCAC ATGACAGCCC AATTTTTTAA AATGGTTATC
TTAAGTCAGG CCAGTTTTAT TTTATTGACC ATGTATATAT
AACATCAGAT ATTTCTAAGA AAGAGAAGAG AACCTGATTG
ATGTCTCTCA TGT (SEQ ID NO. 100)

11E3, contig 1
CCCACCGGGG CCATGGCGTC CGCCGACATC GTCGTGGGCG
GGGTGGCCCA CGGGCGGCCC TACCTCCAGG ATTATTTTAC
AAATGCAAAT AGAGAGTTGA AAAAAGATGC TCAGCAAGAT
TACCATCTAG AATATGCCAT GGAAAATAGC ACACACACAA
TAATTGAATT TACCAGAGAG CTGCATACAT GTGACATAAA
TGACAAGAGT ATAACGGATA GCACTGTGAG AGTGATCTGG
GCCTACCACC ATG (SEQ ID NO. 101)

11E3, contig 2
GTTTATTAAT ATGATCCAAA CAGATTTAGC TTAAGAGATG
AGGACAGGAC ATAAAACAAG ATTTGGCTGA GCTATATAAA
GAAAACCAAA AGAGTGACAA GATTTTCAAT TGGTGACAAT
TTAAAGTCAT TTATTAACTA ATATAAAAAT AAATCTACAA
TAGAACCATG ACTTAAACTG AGCCAGTATG TGGTACATGT
GTCACCCAAA GTCAGCAAGG AACCATTCAG AACTTACACC
CTCCTGGACT GGCTCACACG G (SEQ ID NO. 102)

18H1, 1T3
TTTTTTTTTT TTTTAAACTT AAAAATCAGT TTATCTTAAA
ATTTTTTTGA ATTAGAAGAA ATTGATTTCA CATGAAAATA
TAACATTCTG AAGGTAAGTA TTTTAAACAT TACAACTGTT
(SEQ ID NO. 103)

18H1, 2T3
TGAAAATGCC AAAAATAGTG AACTTAACCT TGTTGGCTAT
GATCTGCTAC ACCAGCCAAC ATTATTGTGC CTTTGCATTT
CACACCAAAA GTTCCAAATG GGTATTTTTT GATGATGCAA ATGTGAAAG
(SEQ ID NO. 104)

18H1, 3T3

TTTTTTTTTT TTTTTTTTTT TAGGGGGACA AATTTAATTT
TTATTCAACT GTAGCATATG ACAAATGAAT TATTATATCT
CCCTGATTTT CTATTTTTCA CGGCACGCCA AGACTATTCA
AAGGGAATTT CTAAAATATC TAAGATATTG ATCATAAAAT
TATATATGTG TATACACATA TACACATACA TATGTATAGT
GATTAACAAC TTTA (SEQ ID NO. 105)

18H1, 5T7

CATGTTTGTC AGGGAGATGC CTGTATATTT TGTGCATTGA
AGACGATATT TGCACAGTTC CAACACAGTC GAGAAAAAGC
ACTTCCCTCA GATAACATAA GGCATGCTCT TGCAGAAAGT
TTCAAAGATG AGCAGCGATT TCAACTTGGC CTTATGGATG
ATGCTGCGGA GTGCTTTGAA AATATGTTGG AGAGGATTCA
TTTTCACATA GTGC (SEQ ID NO. 106)

18H1, contig 1

TAAAAATACT ATCAACATTT AATGTTTCTC TCATAGGTTT
CCAACCTCGG TTCCGGCTTT TACTGCTGCT ATCACAGCTG
TTTCCTCTAT CCCTAGAATC TTGGCTGCTG TTCTGTGTCA
TATCCAGTGC CATTGTCACT CTTAACTTTG CCAGTTATTT
TCTCTCCTGG AGCAAGAATC TGGGATTTAC TTGAACTTAT
TATTTGTGCA GAAGCTMGAC TYTGAGGTAC AACTCGGTCR
TGTTTATATG ATCCTTTTCC TTGA (SEQ ID NO. 107)

03J3, 1T3

GCGCGCCGGC AGCTGCAGGC TCAGGCCATC TCCAGTCTTC
CTGCCGCGCC CGCCTCTCCT CCTGGGATTC TCCTCCTCCT
CCTGGACTTC CCCGCAGCTG CCCACGCCTC GGCGGCCGCC
AGTGCTCCTG GAGTGCAGA (SEQ ID NO. 108)

03J3. 2T3

CTTTTGCGTC GCCAGCCGAG CCACATCGCT CAGACACCAT
GGGGAAGGTG AAGGTCGGAG TCAACGGATT TGGTCGTATT
GGGCGCCTGG TCACCAGGCT GCTTTTAACT CTGGTAAAGT
GGATATTGTT GCCATCAATG ACCCCTTCAT TGACCTCAAC
TACATN (SEQ ID NO. 109)

03J3, 2T7

TTTTTTGGTT GAGCACAGGG TACTTTATTG ATGGTACATG
ACAAGGTCGG CTCCCTAGGC CCCTCCCCTC TTCAAGGGGT
CTACATGGCA ACTGTGAGGA GGGGAGATTC AGTGTGGTGG
GGGACTGAGT GTGGCAGGGA CTCCCCAGCA GTGAGGGTCT
CTCTCTTCCT CTTGTGCTCT TGCTGGGGCT GGTGGT
(SEQ ID NO. 110)

03J3
CTGCCAATTC TTGCTCAATT CTAAGGCAGA TAGACTGTGT
GAGTTCAAAA GATATCCTCT GAAAAGCATC AAAATCTTCC
ACTGTGAACA CATGGGTCTC TGCAGGAGGA GAGGCAATAG
CTTCCAATTC TGAGCGAACG GCATCCTC (SEQ ID NO. 111)

04L3, 11T3
GCAGGGGCTG AAATAACCCA GATGCCCCCA CCCTGCCACA
TACTAGATGC AGCCCATAGT TGGCCCCCCT AGCTTCAGCA GTCACTAT
(SEQ ID NO. 112)

04L3, 17T3
CTGAAATGCA GCTCCCTGTC CAAGTGCCTT GGAGAACTCA
CAGCAGCACG CCTTAATCAA AGGTTTTACC AGCCCTTGGA
CACTATGGGA GGAGGGCCAA GAGTACACCA ATTTGTTAAA
AGCAAGAAAC CACAGTGTCT CTTCACTAGT CATTTAGAAC
ATGTTATCAT CCAAGACTAC TCTACCCTG (SEQ ID NO. 113)

04L3, 24T3
CCTCCGCTTA CAGCTCGCTG CCGCCGTCCT GCCCCGCGCC
CCCAGGAGAC CTGGACCAGA CCACGATGTG GAAACGCTGG
CTCGCGCTCG CGCTCGCGCT GGTGGCGGTC GCCTGGTCCC
GCCGAGGAAG ACTAAGACAA ATCAAGATCT T (SEQ ID NO. 114)

04L3, contig 1
TTTTTTTTYT TTWWTTTYTT TATTCATCAA TAGTATCCGA
AAAGGAAGAA TCAGGAGTTA CAAAAACAAG TTAAATGCAA
TATAGAAGCC TACTAAATAC AAATACAAGT CACAAACACA
TATGCAAGAG AAACTTGTTT AGATTG (SEQ ID NO. 115)

16F1, 1T3
CCGGGCCGGG GAGGCGCGCT CGCTCCGCGC TCCCTTCGCT
CGCTCGTTTC CTCCTCCCTC GGCAGCCGCG GCGGCAGCAG
GAGAAGGCGG CGGCGGCGGC TAGGGATCAG ACATGGCGGC
GGATCTGAAC CTGGAGTGAT CTCCT (SEQ ID NO. 116)

16F1, 1T7
TTTTTTTTTT TTTTTTTTAA GAACATCAAC ATTTATTTAA
CATGATAAAA AAAGAAATGA GATATGAACA TTTGCATTTA
AACAATAGTA AGTAGCCTTT AATACATTAC ATGTGCTCAT
TGTATAATAT ATACACAATG AACATAATTA CATTTGTACA
CAAACTAAGT ACCGGATTTG GAAACCTGCT TATTGCTGTA
CACATGTATT CCAATG (SEQ ID NO. 117)

16F1, 3T3
CACCACGATC AAAAGGGACA AGCATCAAGC ACGCAGCAAT
GCAGCTCAAA ACGCTTAGCC TAGCCACACC CCCACGGGAA
ACAGCAGTGA TTAACCTTTA GCAATAAACG AAAGTTTAAC
TAAGCTATAC TAACCCCAGG TTGGTCAATT TCGTGCCAGC
CACCGCGTCA CACGATTACC AAGTCATAGA G (SEQ ID NO. 118)

16F1, contig 1
TTTTTTTTTT TTTTTTTTTK YTYWTTTYYT TGTTTTTTTG
ATTGTTTTGG ATCTCTGGTT TAATTAGCAC TCTATGGTTG
GGAATGTTAT TGGTTTCTTT AGTTGGTGCA TTTTCAGATG
TAATCTTGTC CACTCTTYTC ACAGGTTCTG TCTGTACTAG
GGCAGCATCT AACATGGCTT TCATCCACAA CTCCATTTCC
TTTCCTGTAT CAGTGCAGAA AAAAGG (SEQ ID NO. 119)

16F1, contig 2
CGGATCTGAA CCTGGAGTGG ATCTCCCTGC CCCGGTCCTG
GACTTACGGG ATCACCAGGG GCGGCCGAGT CTTCTTCATC
AACGAGGAGG CCAAGAGCAC CACCTGGCTG CACCCCGTCA
CCGGCGAGGC GGTGGTCACC GGACACCGCG GCAGAGCACA
GATTTGCCTA CTGGCTGGGA AGAAGCATAT ACTTTT
(SEQ ID NO. 120)

07L1, 2T3
AGATGGTCCA GCTGCCAGGA CTACTTTGGC AGGCAGCGTG
CTACAGGACG AAAATGTAAG AGAAGTCTAT TAAGGCTGGA
CAGCCCAGGG TTATTTATAC TCTCTCAGCC CCAAGTCCCC
CGGACTAAAG ACCTAAAGGC TGATTGACTC ATTCCTGATT
GATTTAATGG AAAGTCTCCC ACCCCATCAT CATTTGCCAG AGTAC
(SEQ ID NO. 121)

07L1, 2T7
CAAAACTCAG CAGTGCTTCT GGTGCTGGTG ATCAGTGCTT
CTGCAACCCA TGAGGCGGAG CAGAATGACT CTGTGAGCCC
CAGGAAATCC CGAGTGGCGG CTCAAAACTC AGCTGAAGTG
GTTCGTTGCC TCAACAGTGC TCTACAGGTC GGCTGCGGGG
CTTTTGCATG CCTGGAAAAC TCCACCTGTG ACACAGAT
(SEQ ID NO. 122)

07L1
TTAGATGCAA GAAGATGCAG GCTCAAAGTC TGGTTGGACA
GCCAGGCTCA AGCAATTTGG TAAATGTGTC GGAAAGAAAA
TTAGACATTG GAGGATCAAG ACCATAAGAC ACTAGCTCAT
TAGAGATCAA GAATTCAAAT GTGACATTCA TATTCGTCC
(SEQ ID NO. 123)

10F1
ATTTTAAAAG AATACTGTAC TGGAATGGGT TAACATCATC
ATCTTTGGCA TCCT (SEQ ID NO. 124)

10F1, 2T3
GACATTCGCC CTGATATAAA AGATGATATA TATGACCCCA
CCTACAAGGA TAAGGAAGGC CCAAGCCCCA AGGTTGAATA
TGTCTGGAGA AACATCATCC TTATGTCTCT GCTACACTTG
GGAGCCCTGT ATGGGATCAC TTTGATTCCT ACCTGCAAGT
TCTACACCTG GCTTTGGGGG GTATTCTACT ATTTTG
(SEQ ID NO. 125)

10F1, 5T3
TCGCACTTTG CCCCTGCTTG GCAGCGGATA AAAGGGGGCT
GAGGAAATAC CGGACACGGT CACCCGTTGC CAGCTCTAGC
CTTTAAATTC CCGGCTCGGG GACCTCCACG CACCGCGGCT
AGCGCCGACA ACCAGCTAGC GTGCAAGGCG CCGCGGCTCA
GCGCGTACCG GCGGGCTTCG AAAC (SEQ ID NO. 126)

10F1, 5T7
TTTTTTAAAT TACAACACTT TATTGCAGCA TCGGCAAAGG
TCAGATTTCT GAAGCTGGTG AAGATTGGGC AGCATTTCCA
TGTGAAATGT TACAACTTTA CAAGTTTTGT TTTTTATTTA
AATCTACATG CAGAAACTGA AACATGGTAA AAGAAAAAAT
GCAAAATAGC TAGAAAAAAA GATGTAATCA AGTTGTCGCA
TACAGATGTG CTCTCCG (SEQ ID NO. 127)

10F1, 6T3
CTTGTCTGAC CTAGAAGCTG AGAAACTGGT GATGTTCCAG
AGGAGGTACT ACAAACCTGG CTTGCTGATG ATGTGCTTCA
TCCTGCCCAC GCTTGTGCCC TGGTATTTCT GGGGTGAAAC
TTTTCAAAAC AGTGTGTTCG TTGCCACTTT CTTGCGATAT
GCTGTGGTGC TTAA (SEQ ID NO. 128)

10F1, contig 1
TTTTTTCAGA TTCACTTCAC TTTTATTATG AACAAACACA
ATCTCAGATT AGTACAATTA GCTTCAGAGT TGATATTAAT
AGAAATTATT CCAAAATTAT TCTTGTCACA AGTAACTACT
ATATCCCACA TAAAAAGGGA AAAAATCCCA CCCAATCACA
GAAAAGGCAT CCTCTGTATG TTTCCGTGGC AATGCGTTGT
TTATGTATTC TCAAATTTTG TCTGGCTAGT TATC
(SEQ ID NO. 129)

05D1

TTGGTGAGGG TGGGGAGGAG TGCTTCGAAG GGAGAAGCCC
CAGCAAGATT TATTCCTTTT TGCTTCTTCT TCTCCCTGTC
CCTGCCATAA CCATGAAGCC TTGAACAAAC CACCCAAATC
TCAGGATCTT AGTGTTTTCT CTGTAAATTG TAATATGAAC
TTATAAAGAT CCTCCATTGC TGATAGTCTC AGGTTCTGTG
AGTAACAGCA AAAAAACTTT GTATCTAACT TCAACCAGAG
CAGGCTGTAC CCTTAAGCTC T (SEQ ID NO. 130)

The sequences shown above can be used in gene therapy to produce RNA directly in cells or tissues, as nucleic acid probes in diagnostic methods, as nucleic acid primers, and as components of recombinant DNA cloning and/or expression vectors. Further, those of skill in the art will recognize that the complete coding sequence of a gene corresponding to a genetag of the invention can be isolated once the sequence of the genetag is known. Thus, those in the art could use the above sequence information to isolate the coding sequence of the gene and to express the mRNA or protein encoded by the gene. Diagnostic and therapeutic applications for an expressed mRNA and corresponding protein are known in the art and are applicable to the practice of the present invention. For example, antisense oligonucleotides or ribozymes can be designed to target the mRNA, and antibodies can be generated to target the protein. Such antisense oligonucleotides, ribozymes, and antibodies can be used for the detection of senescence-related genes or to prevent the deleterious expression of such genes, as described above.

As noted above, probes and/or primers comprising the sequences shown in Table 4, or those shown in Table 6, or sequences from other senescence-related genes identified according to the methods of the present invention can be used in diagnostic methods to detect the presence of young or old cells in a tissue or other sample. Several probes that were identified to be young or senescent

specific by Northern analysis have been used in in situ analysis of human skin tissue sections. These probes can be used to identify senescent cells or young cells in tissue (in vivo; in situ). Probes for Collagen 1α1, Collagen 1α3, and for MnSOD were used in a commercially available in situ hybridization protocol (SureSite™ II System Manual, Novagen). Preliminary results show that the probes for Collagen 1α1 and Collagen 1α3 detect RNA in dermal fibroblasts in human skin sections of two young donors (<15 years age), while probes for MnSOD detects RNA in dermal fibroblasts in human skin sections of two old donors (>69 years age). These results confirm the expectation that young specific genes detect cells in tissue from young, but not normal old, donors and that senescent-specific genes detect cells in tissue from old, but not normal young, donors. These findings support the belief that replicative senescent dermal fibroblast cells accumulate with age in human skin.

This *in situ* data illustrates the value of the nucleic acid primers and probes of the invention. Primers and probes are oligonucleotides that are complementary, and so will bind, to a target nucleic acid. Although primers and probes can differ in sequence and length, the primary differentiating factor is one of function: primers serve to initiate DNA synthesis, as in PCR amplification, while probes are typically used only to bind to a target nucleic acid. Typical lengths for a primer or probe can range from 8 to 20 to 30 or more nucleotides. Modified, synthetic, and/or non-naturally occurring nucleotides can also be used in whole or in part in the oligonucleotides of the invention. A primer or probe can also be labelled to facilitate detection (i.e., radioactive or fluorescent molecules are typically

used for this purpose) or purification/separation (i.e., biotin or avidin is often used for this purpose).

Depending in part on the length and/or intended function of the primer, probe, or other nucleic acid comprising sequences from a senescence-related gene, expression plasmids of the invention may be useful. For instance, recombinant production of RNA corresponding to a genetag or senescence-related gene of the invention can be carried out using a recombinant DNA expression plasmid of the invention that comprises a nucleic acid comprising the nucleotide sequence of the genetag positioned for transcription under the control of a suitable promoter. Host cells for such plasmids can be either prokaryotic or eukaryotic, and the promoter, as well as the other regulatory elements and selectable markers chosen for incorporation into the expression plasmid will depend upon the host cell used for production.

One important use of probes derived from the genetags and corresponding genes of the present invention relates to a method for screening compounds to identify compounds that can alter gene expression in senescent cells, which method comprises: (a) contacting senescent cells with a compound; (b) determining mRNA expression patterns in said senescent cells; and (c) correlating an alteration in mRNA expression of a senescence-related gene with identification of a compound that can alter gene expression in senescent cells. Preferably, the determination of mRNA expression pattern involves a determination of mRNA expression levels of two or more senescence-related genes. Thus, this screening method identifies compounds with the capacity to reverse, partially reverse, or modulate the pattern of gene expression that is altered as a consequence of senescence. The present invention also encompasses the compounds identified by this method and the use of those compounds to alter gene expression in senescent cells.

Such screening can also identify compounds that activate young-specific genes or prevent cells from entering a senescent state. In this method, the novel oligonucleotide probes of the invention serve as indicators of whether a test compound can alter the expression levels of a senescence-related gene.

Compounds ideally suited for testing in this method include compounds identified in primary screens based on the expression of a specific senescence-related gene product. In general, the basic format of the screen is as follows. Senescent cells are cultured in 96-well microtiter plates. After an incubation period, i.e., three days in culture, the medium will be removed and assayed for one or more senescence markers, providing a "before treatment" baseline. The medium will be replaced with fresh medium containing a test agent or its vehicle. The cells will be cultured for an additional period, i.e., two to four days or more in culture, in the presence of the test agent. The cells and/or medium will then be assayed for the senescent markers ("after treatment" measurement). Samples that normalize the senescence markers can be presented to cultures of young cells and their effects measured in a similar fashion. Compounds that act selectively on the senescent cells will proceed to additional screening.

As noted in Table 2 and in the scientific literature, a number of known genes are senescence-related genes. For instance, the activity of ß-galactosidase is elevated in senescent fibroblasts. (See, Cristofalo, et al., 1975, Mech. Ageing Dev. 4:19-28; and Dimri et al., 1995, Proc. Natl. Acad. Sci. USA, 92 (in press)). Consequently, one can first conduct a primary screen of test compounds to determine whether that compound inhibits ß-galactosidase activity in senescent cells. In one embodiment of this screen, fibroblasts are grown to senescence, plated in 96 well plates, and incubated with

a test compound. At the end of the incubation period, cells are analyzed for enzyme activity using a colorimetric assay based on the ability of the enzyme to cleave a colorless substrate into a colored reaction product. Compounds identified in this screen (see Example 14, below) as active compounds will then be tested in a secondary assay to determine that the active compounds are inhibiting the senescence-specific increase of activity of the enzyme and not merely inhibiting the enzyme itself. Other primary screens can be conducted using the senescence-related genes identified in Table 2, above, identified according to the methods of the invention, or known from the scientific literature. For instance, one could conduct a primary screen to identify compounds that have the capacity to induce the down-regulation of collagenase activity, an enzyme that is known to be elevated in senescent fibroblasts.

Compounds active in these screens can then be tested according to the screening method of the invention to determine whether the compound inhibits the expression of other senescence-related, specifically old-related, genes or activates the expression of young-related genes, or both. The method can employ Northern analysis to examine the effects of the lead compounds on panels of genes that show altered expression or abundance in senescence as indicated by EDD. Based on the results of this screen, one can determine which compounds normalize the expression of those genes that are altered in senescence and believed to contribute to age-related pathologies. Furthermore, it will be possible to determine the level at which the compound acts to reverse the pattern of altered expression. Complete reversal to a young pattern of gene expression would suggest that a single common mechanism is involved. Reversal of defined groups of genes would indicate that several mechanisms are operating and that each is affecting a different bank of

genes. A compound may also act to modulate the activity of individual genes, suggesting the absence of a common mechanism. This information will in turn influence primary screening strategy. If, for example, all active compounds seem to reverse the altered expression of batteries of genes, or of only individual genes, then the screen can be expanded so that many more markers, including members from each of the putative batteries, if appropriate, are included.

Screens such as those described above can be based on many different cell types. For example, several types of skin models are useful for such screening methods. Examples of useful skin models include the wounded dermal model (see, Genever, et al., 1993, Exp. Dermatol. 2: 266-273), keratinocytes proliferated and differentiated on collagen sponge containing fibroblasts (see, Maruguchi et al., 1994, Plast. Reconstr. Surg., 93: 537-544), a cultured skin substitute composed of fibroblasts and keratinocytes with a collagen matrix (see, Kuroyanagi, et al., 1993, Ann. Plast. Surg., 31: 340-351), reconstituted non-crosslinked collagen, or reconstituted collagen chemically crosslinked with glutaraldehyde, aluminum alginate or acetate (see, Middelkoop et al., 1995, Cell Tissue Res., 280: 447-453), and human keratinocytes grown on a polyglactin mesh-cultured fibroblast dermal substitute (see, Hansbrough, et al., 1993, J. Burn Care Rehabil. 14: 485-494).

In addition, the gene aldehyde dehydrogenase 1 (ALDH-1), found by EDD to be a quiescent- upregulated gene, can also be used in a screen, ALDH-1 is believed by those in the art to have a 17 beta dehydrogenase activity. The down-regulation of this gene in senescent cells could therefore lead to altered steroid metabolism (i.e., more active androgen). Since the cells in the prostate, hair follicle, and hair sebaceous gland respond to androgen,

this increased androgen activity could lead to benign prostatic hyperplasia, male pattern hair loss, and sebaceous gland hyperplasia (conditions known to be age-related). The gene ALDH-1 can be used in a screen to find agents that inhibit the oxidase, but not the reductase activity of the enzyme, thereby allowing the enzyme to more efficiently metabolize steroids present in cells.

Using cultured cells for screening requires that a number of technical challenges be met. First, the cells must be kept viable during the screening. Second, the metabolism of the cells must not be perturbed by the assay conditions. Third, particularly in a multiple-day screen, it is vital that sterility be maintained. Culture conditions that will produce old senescent and young quiescent and mitotic cells must be carefully selected, taking into consideration the following criteria for senescence: (1) the cells typically will exhibit a change in the morphology that is characterized by the enlargement and flattening of the cell as it reaches senescence; (2) the cell will irreversibly leave the cell cycle and will be incapable of proliferation for a minimum of three weeks as measured by the population doubling time of the cells in culture; and (3) the cells typically will exhibit a nuclear labeling index below 1%, as measured by the incorporation of a labeled DNA precursor in the nuclei over a period of 24 hours (see Example 1, below). As the cells approach senescence, their generation time increases. This means that the interval between passaging cells is constantly varying and must be determined by continuously monitoring cell density. The time at which cell replication ceases (replicative senescence) must be determined.

Cell-based screens have traditionally been labor intensive and so have not often been used for high-throughput screening. However, the present method is

amenable to high-throughput screening. Liquid handling operations can be performed by a Microlab 2000™ pipetting station (Hamilton Instruments). Other equipment needed for the screen (e.g. incubators, plate washers, plate readers) can either be adapted for automated functioning or purchased as automated modules. Movement of samples through the assay will be performed by an XP™ robot mounted on a 3m-long track (Zymark).

Through these screens, libraries of synthetic organic compounds, natural products, peptides, and oligonucleotides can be evaluated for their capacity to modulate the expression of genes that reflect or contribute to the disease process. Specifically, compounds can be identified that will down-regulate genes that are up-regulated during senescence or, conversely, will increase the expression of genes that are down-regulated during senescence. Active compounds can be optimized, if desired, via medicinal chemistry. Initially, one can define a pharmacophore(s), using modern computational chemistry tools, representative of the structures found to be active in the high throughput screens. Once a consensus pharmacophore is identified, one can design focused combinatorial libraries of compounds to probe structure-activity relationships. Finally, one can improve the biopharmaceutical properties, such as potency and efficacy, of a set of lead structures to identify suitable compounds for clinical testing.

Thus, the present invention provides novel methods for identifying senescence-related genes, methods and reagents for identifying senescent and young cells and for distinguishing senescent from young cells in tissue, and compounds and therapeutic methods for treating diseases and conditions resulting from cell senescence targeting senescent cells. The following examples describe specific aspects of the invention to illustrate

the invention and provide a description of the present methods for those of skill in the art. The examples should not be construed as limiting the invention, as the examples merely provide specific methodology useful in understanding and practice of the invention.

Example 1

Determining Mitotic Index of Cells with Immunohistochemical Staining

This assay allows one to quantitate the fraction of cells in S phase of the cell cycle via detection of incorporated 5-bromo 2-deoxyuridine into DNA and can be used to determine when cells are senescent.

A. Preparation and Treatment

1. Grow putative senescent cells on sterile coverslips (Corning No. 1, 18 mm sq.) to 60% confluence. Allow cells to "recover" for one day before treating them with 5-bromo-2'-deoxyuridine (BrdU). Do not wash cells just prior to incubation with BrdU; this will slow the growth of the cells during the incorporation phase of the procedure.

2. Add BrdU (Sigma #B-5002) at a final concentration of 10 mM to cells in growth media. Keep the BrdU shielded from light during the addition step. Incubate the cells for a defined time period (typically 2 to 24 hours) in the dark at 37°C. One should have 1 mM stocks of BrdU dissolved in PBS and aliquoted into light protective tubes (or wrapped in foil protected from light) already made prior to performing the assay.

3. After the BrdU treatment, aspirate media into a waste bottle containing bleach, and wash the cells 3 times with PBS. After the third wash, add 3 ml of PBS to each well.

B. Fixation

1. The plates should contain 3 ml of PBS in each well and be kept on ice. Add 3 ml of fix solution, which is ice cold methanol:glacial acetic acid (3:1), to each well (on ice).

2. Remove 3 ml from each well and discard. Add 3 more ml of fix solution to each well.

3. Repeat step 2 two more times.

4. Remove 5 ml from each well and add 6 ml of fix solution to each well. Leave the plates on ice for 15 minutes; then, repeat this step once again.

5. Remove coverslips from plate and allow to air dry overnight or for several hours. The solvents should be completely evaporated. Store coverslips in a covered box in the dark for staining the next day. If coverslips are to be kept longer than one day before staining, store the coverslips frozen in a dessicator under an atmosphere of N_2.

C. Immunohistochemical Staining

1. Treat fixed cells with 0.01 N NaOH for 3 minutes to denature DNA and expose the antigen. Do not overtreat cells with base; the cells will fall off the coverslips.

2. Neutralize the base with PBS at pH=8.5. Wash the cells two times with PBS to remove all of the base.

3. Block the cells with 1.5% Horse Serum (Vector Labs #S-2000) in PBS (use serum from species in which secondary antibody was generated) for 15 minutes.

4. Carefully aspirate or tilt coverslips to drain horse serum from cells. Do not wash the coverslips at this point.

5. Add 400 ml of anti-bromouridine monoclonal antibody (Sigma #B-2531, made in mouse, IgG) at a dilution of 1/500 in 1% BSA, 0.05% Tween 20, in PBS, to cover cells completely. Incubate for 2 hrs. at room temperature in a humid chamber away from light.

6. Rinse cells in PBS three times.

7. Add 400 ml of secondary biotinylated horse anti-mouse IgG (Vector Labs #BA-2000) for 30 min. at room temperature in a humid chamber free from light. The antibody should be diluted in 1.5% horse serum in PBS at 10 mg/ml.

8. Wash cells three times with PBS.

9. Incubate cells with 400 ml of 30 mg/ml Fluorescein Avidin D (Vector Labs A-2001) in 10 mM HEPES, 0.15 M NaCl, pH=8.5, for 20 min. at room temperature in a humid chamber protected from light.

10. Wash the cells with PBS 3 times.

11. Add 4',6-diamidino-2-phenylindole, DAPI, (Sigma #D-9542) at a concentration of 1 mg/ml in 2X Standard Saline Citrate (SSC) buffer for 5 minutes to stain nuclei. Standard Saline Citrate is prepared by first making 20X SSC as follows: dissolve 175.3 g NaCl and 88.2 g sodium citrate in 800 ml of water; adjust pH to 7.0 with 10 N NaOH; and adjust volume to 1 liter with water; then, dilute 20X SSC to 2X SSC by adding 10 ml 20X SSC to 90 ml of distilled water. It is convenient to have stock solutions of DAPI prepared and stored in light protected tubes at 1 mg/ml dissolved in distilled water.

12. Aspirate DAPI, and rinse cells 3 times with PBS.

13. Mount coverslips on slides using Vectashield™ (Vector Labs, #H-1000) mounting medium to reduce quenching of FITC. Do not use too much of the mounting medium, as it will not dry completely. Seal coverslips to slides using clear nail polish, and allow to dry for 5 minutes.

14. View slides under the fluorescent microscope.

D. Calculation of Mitotic Index

The mitotic index is equal to the ratio of the number FITC labelled cells to the number of DAPI labelled cells in a given field.

E. Staining with Acridine-Orange to Identify Senescent Human Diploid Fibroblasts

In another method for staining cells to detect senescent cells the stain acridine orange is used. Acridine orange (AO) is a fluorescent dye which has unique spectral properties. In this method, growing cells are stained by adding AO in aqueous solution directly to culture medium and incubating the cells at 37°C (5% CO_2) for approximately 30 minutes. The cells are then viewed by epifluorescence microscopy using a 488nm excitation filter, 520 long-pass barrier filter ("FITC Filter Sets"). The fluorescence emitted from senescent cells differs from that of replicatively young cells in that (1) there is an increase in total fluorescence in the cytoplasm and a shift in emission spectrum towards longer wavelengths with senescent cells, and (2) there is a shift in emission spectrum towards longer wavelengths in the nuclei of senescent cells. Further, there is a change in the pattern of fluorescence in the nuclei of senescent cells which is consistent with the structural change in chromatin organization with senescence. This pattern can be diagnostic of senescent cells. This staining method can be applied to the analysis of tissues *in situ*.

Example 2

Cell Culture and RNA Preparation

Human fibroblast cells are split at the appropriate density according to standard tissue culture techniques. Cells are grown in DMEM medium plus 10% bovine calf serum (BCS). The last split before RNA isolation is 1 to 8 for young cells and 1 to 2 for senescent cells. After the cells are split and seeded in DMEM medium plus 10% BCS, two protocols are followed.

(1) If mitotic cells are required, cells are grown at 37°C in DMEM medium plus 10% BCS for two days. Then, RNA is isolated (see below).

(2) If quiescent cells are required, the DMEM medium plus 10% BCS is aspirated 4-8 hours after the seeding, when the cells have attached. The medium is replaced with DMEM medium plus 0.5% BCS, and the cells are grown for 3 days at 37°C. The medium is changed with fresh DMEM medium plus 0.5% BCS, and the cells are grown for 2 more days at 37°C.

For RNA isolation, cells are quickly washed once with PBS; then, the wash is aspirated completely, and about 1.5 to 2 ml of GITC solution (200 ml of GITC solution are prepared by adding 94.53 g of guanidine isothiocyanate to 1.67 ml of 3 M sodium acetate (pH=6), adding DEPC-water to 200 mL, sterile-filtering (0.22 µm filter), and adding, in a fume hood, 1.67 ml of beta-mercaptoethanol) is added to a 15 cm plate. The cells lyse in this solution. After a few minutes of rocking the plate back and forward, to cover all areas, the (slimy) lysate is collected and prepared for $CsCl_2$ centrifugation.

Example 3

Spinning Guanidine Isothiocyanate (GITC) RNA Isolation

Before beginning this procedure, make sure the RNA+GITC and CsCl are at room temperature. The CsCl solution is prepared by adding 95.97 g of CsCl to 0.83 ml of 3 M sodium acetate, pH=6, and adding water to a final volume of 100 ml.

1. Use Ultraclear™ 14 x 89 mm polycarbonate tubes (Beckman 34059).

2. Add 4 ml of 5.7 M CsCl to the bottom of the tubes.

3. Gently, without disturbing the interface, add 7 ml of the Guanidine Isothiocyanate + RNA to these tubes.

4. Balance the tubes, and then place the tubes in the swinging buckets that are part of the SW41 rotor.

5. Spin at 32K for 20 hours at 20°C.

6. After the spin, aspirate the supernatant (RNA is pelleted at the bottom of the tube).

7. After inverting tubes and letting them drain for about 30 minutes, cut off the bottoms of the tubes.

8. Resuspend the pellets in 180 µl of diethylpyrocarbamate-treated deionized H_20 (DEPC-water).

9. Then do a second wash of the tube bottoms with another 180 µl.

10. Pool these washes (total of 360 µl) and add to them 40 µl of 3 M sodium acetate, pH=6 (filter-sterilized, made with DEPC-water).

11. Vortex and add 1 ml of cold 100% ethanol, mix, and place at -80°C for at least 30'. To precipitate RNA, use 2.5 volumes of ethanol.

12. Spin for 30' at 14K at 4°C. Carefully aspirate off the ethanol. Don't let the tubes evaporate, because this will leave salt deposits behind.

13. Resuspend in DEPC-water. Decide on volume based on the pellet size.

14. Take the O.D. (260), i.e., 1 µl in 100 µl and use a 100 µl quartz cuvette.

To check the RNA for quality control, one can run a gel, using the following procedure.

1. Heat 1-2 µg of RNA in 10 µl of DEPC-water at 70°C for 2 minutes. Incubate at room temperature, and add a standard loading dye to the sample.

2. Run a 1.2% agarose gel made with 1xTAE buffer made with DEPC-water.

3. Run the gel hot and fast, i.e., 150 volts for a 5" x 7" gel or 75 volts for a mini-gel.

Example 4

Enhanced Differential Display

A. Synthesis of first-strand cDNA

The annealing reaction is conducted by mixing 1 mg total RNA with 2.5 ml of 20 μM 3'-primer (dT_{12} mer); and 9.5 ml of DEPC-H_2O. The resulting solution is heated for 10 minutes at 75°C, then cooled on ice for 7 minutes, and then spun to collect the mixture at the bottom of the tube.

The elongation reaction is conducted by adding to the tube 5 ml of 5X first strand synthesis buffer; 1 ml RNAsin (Promega or Pharmacia); 2.5 ml of 0.1 M DTT; 2.5 ml of 0.25 mM dNTP; and 1 μl of reverse transcriptase (SuperScript™ II RT, BRL). The resulting solution is incubated for 70 min. at 37°C. Then, the solution is heated to inactivate the enzyme by incubating the mixture for 10 min. at 95°C. The reaction mixture can be stored at -20°C for later use.

B. PCR amplification of cDNA

The reaction mixture is prepared using 1 ml of cDNA (3' primer carried over from cDNA); 2 ml of 10X PCR buffer (500 mM KCl, 100 mM Tris pH 8.3, 20 mM MgCl); 1.5 ml of 0.1 mM dNTP; 1.25 ml of 20 mM 5' primer; 1 ml of a 1 to 5 dilution of a-^{32}P dATP; 0.5 ml of Taq polymerase; and 12.75 ml H_2O. About 70 ml of mineral oil are layered on top of the reaction mixture, which is briefly centrifuged to collect the reaction mixture at the bottom of the tube.

The PCR machine (Perkin-Elmer) is programmed to conduct 4 cycles of 94°C for 45 sec.; 41°C for 60 sec.; and 72°C for 60 sec., and then 18 cycles of 94°C for 45 sec.; 60°C for 45 sec.; and 72°C for 120 sec. The tubes are centrifuged briefly to collect the reaction mixture at the bottom of the tube and can be stored at 4°C.

C. Differential Display Gel Analysis

1. Mix 3 ml of PCR product with 2 ml of running dye (formamide dye).

2. Heat samples for 3 min. at 90°C, pulse spin, and load on a 6% sequencing gel (see part D) in 0.6X TBE and run gel at 2000 V (current of ~50 mA).

3. Run the gel until the second dye reaches the bottom (this can be varied depending on what size range of bands one wants to compare).

4. Dry gel, and when the gel is dried, tape the gel and the film together, punch holes at the three corners of the gel, and expose to film overnight.

D. Sequencing gel

To prepare the sequencing gel, mix 36 grams of urea with 11.25 ml of 40% acrylamide/bis solution (19:1) and 4.5 ml of 10X TBE, and add DEPC-H_2O to 75 ml, and allow the components to go into solution. Then, filter the mixture through Nalgene 100 ml disposable filterware (CN), and add 330 ml of 10% ammonium persulfate and 33 ml of Temed, and pour the gel immediately.

Example 5

Removing Differentially Displayed Bands

1. Line film up with gel.

2. Poke holes through the film and gel on each side of the band with an 18 gauge needle.

3. Draw a line between the dots on the gel representing the band to be removed (this makes it easier to see the saran wrap in later steps).

4. Cut band out with a clean razor blade and manipulate band with clean forceps (rinse both items with water in between retrieving each band).

5. Use a new razor blade every six bands.

6. Place band into a 1.5 ml Eppendorf™ tube and add 1 ml of TE buffer. Let soak for 15 minutes.

7. Aspirate the buffer and separate the saran wrap and paper from the gel slice.

8. Then add back 1 ml of TE buffer and aspirate again immediately (to dilute out urea in the gel slice).

9. Add 40 µl of TE buffer + 100 mM NaCl

10. Heat for 10 minutes at 95°C (boiling water bath).

11. Let tubes cool overnight at room temperature.

12. Pulse spin at 14K and remove 5 µl for PCR amplification.

13. Add 5 µl 10X PCR buffer, 2.5 µl 1 mM dNTP, 3 µL 20 µM 5'-primer, 3 µL 20 µM 3'-primer, 1 µl Taq polymerase, DEPC-water to 50 µl.

14. Run PCR (Perkin-Elmer machine) for 25 cycles, each cycle consisting of 94°C for 45"; 60°C for 1'; and 72°C for 1'; and then incubate at 70°C for 15'.

Example 6

PCR Sequencing Differentially Displayed Bands

1. Heat gel slice at 95°C to liquefy.

2. Remove 3.5 µl and place into a 1.5 ml tube containing 1.5 µl of the appropriate 20 µM 3'-primer.

3. Add 5 µl of the dideoxynucleotide termination mix to the wells in the microtiter dish.

4. Make up a cocktail containing 10x sequencing buffer, ^{32}P-alpha-ATP, Taq polymerase, and water.

5. Add 18 µl of this reaction cocktail to the tube containing the PCR-amplified band and primer.

6. Heat at 95°C for approximately 20" immediately before adding 5 µl of this cocktail to the appropriate termination mixes in the microtiter dish.

7. Overlay with 20 µl of mineral oil.

8. Add a drop of mineral oil to each of the wells in the PCR machine before inserting the microtiter dish.

9. Program the machine to conduct a 95°C soak for 5′ and 30 cycles, each consisting of 95°C for 30″; 60°C for 30″; and 72°C for 1 min. Check to be sure that the sample probe heats up quickly enough.

10. When finished add 5 µl of stop mix.

11. Denature samples in PCR machine by soaking 5 minutes at 95°C. Immediately load samples on the gel.

Example 7

Cloning Differentially-Displayed Bands in Bluescript SK+

A. Digestion of bands with HindIII

Choose 4 old- or young-specific bands to clone. Digest 5 µl of each solution of band DNA, previously PCR-amplified from an acrylamide gel slice of a DD gel. The reaction mixture comprises: 5 µl of band DNA, 5 µl of 10X restriction buffer B (Boehringer Mannheim); 39 µl of deionized H_2O; and 1 µl of restriction enzyme HindIII (10 U/µl, Boehringer Mannheim) in 50 µl total reaction volume at a temperature of 37°C for 2-3 hrs. After digestion, heat-inactivate the enzyme by incubation at 70°C for 20 minutes.

B. Preparation of Bluescript vector for cloning

It is useful to prepare a stock solution of ~50 µg of HindIII-digested pBluescript (Stratagene) at a concentration of ~0.25 µg/ml, which can be stored in the freezer and used as a stock to aliquot from when preparing fresh calf-intestinal alkaline phosphatase-treated (CIPed) vector every 2-3 weeks. If more HindIII-digested pBluescript has to be prepared, digest 30-50 µg of pBluescript with HindIII. Do not place all of this DNA in one tube, but rather use several tubes, each containing 5 µg in a reaction volume of 20 µl. Digest at 37°C for at least 3 hours to ensure that digestion is

complete. Digestion must be complete, or too many blue colonies will appear after transformation. After digesting, run 1 ml of each digest on a 1% agarose gel to verify that digestion is complete. If digestions are not complete (i.e., if you see an additional band indicative of supercoiled DNA), combine all of the digests in one tube, add 5-10 µl of HindIII (do not add any more buffer) and incubate at 37°C for 2-3 hrs. Check 2-4 µl on a gel to ensure again that digestion is complete.

Prepare fresh CIPed HindIII-digested pBluescript SK+ every 2-3 weeks by reacting 20 µl, i.e., ~5 µg, of pBluescript (HindIII-digested) with 1 µl CIP (1 U/ml, Promega), 3 µl of 10X CIP buffer (Promega), 6 µl of deionized H_2O in a total volume of 30 µl at 37°C for 1 hr. Add EDTA to a final concentration of 5 mM (i.e. add 0.5 µl of 0.31 M EDTA) and incubate at 70°C for 30 min. and then phenol extract the solution once. Then, add 1/10 volume of 3 M NaAcetate (pH=7.0) and 2 volumes of 100 % EtOH. Place on dry ice for 20 min. or overnight at -20°C. Spin down the DNA in a microfuge by 10 min. of centrifugation, and wash with ~200 µl of 70% EtOH (ice cold). Do another 5 min. spin, and dry the DNA pellet in a dessicator or speed-vacuum centrifuge. Resuspend the DNA (~5 µg) in 25 µl of deionized water (dH_2O) to a final concentration of ~0.2 µg/ml. Check 1 µl of this solution on a 1% gel to make sure the DNA is recovered in good yield. Use ~1 µl per ligation reaction.

C. Gel Purification of HindIII-digested band and ligation into HindIII-digested, CIPed vector

After digesting bands with HindIII and heat-inactivating the enzyme, load the entire digestion reactions on a 2% low melting point agarose gel. To prepare the gel, follow the procedure below.

Note that an 8" x 10" gel box that will house a 30 well comb typically holds 300 ml of gel mix.

1. For a 2% gel, add 6 g of seaplaque low melt agarose to 300 ml of 1x TBE buffer. Note that 1% is 1 g/100 ml of gel.

2. Heat the solution in the microwave at level 7 for about 4 min. When finished, place the solution in the 65°C water bath to cool to a temperature suitable for handling the flask.

3. Add 3 µl of 5 mg/ml EtBr to the gel, mix by shaking, and add 4 µl to the 1x TBE running buffer.

4. Load .5 µl of the ØX-174 RF DNA digested with HaeIII (Pharmacia) on both sides of your samples.

5. Run gel between 100 and 150 volts for about 1.5 to 2 hours.

Note: when adding loading dye to samples, add 1 µl of dye to 5 µl of sample.

Load 2 µl of the 123 bp DNA ladder (BRL) as a marker. Take a picture of the ethidium bromide-stained, and UV-irradiated gel, and confirm that the size of the bands on the gel matches the sizes indicated on the data sheets. Cut out bands using coverslips and place the cut out bands into Eppendorf™ tubes. To remove the bands from the gel, follow the procedure below.

1. Clean a 365 nm UV light box by wiping the surface with ethanol.

2. Place the gel on the UV light box, and take a photograph using a hand-held camera with the aperture set on 8 and the time (which the shutter stays open) on B (hold trigger down for approximately two seconds).

3. Examine the photograph to make sure the bands are the proper size.

4. Cut bands out using glass coverslips, and remove with an ethanol-sterilized spatula.

5. Place the band in a labelled 1.5 ml Eppendorf™ tube, and discard the glass coverslip, in addition to re-sterilizing the spatula.

One should wear a face shield, lab coat, and gloves when cutting out the bands to protect against UV exposure.

Set up ligation reactions by mixing 2 µl of Band DNA (melt down agarose at 65°C, 10 min. before adding) with 1 µl of CIPed, HindIII-digested Bluescript (~0.2 µg/ml), 2 µl of 10 mM ATP, 2 µl of 10X One Phor-All™ buffer (Pharmacia), 12 µl of dH2O, and 1 µl of T4 DNA ligase (Pharmacia) in a total volume of 20 µl, and incubate at 37°C for 2-3 hrs.

D. Transformation of subcloning efficiency DH5alpha competent cells

To prepare the competent cells (DH5alpha cells are available from BRL), follow the procedure below.

1. Grow an overnight culture in 3 ml of media.

2. Place all of the overnight culture into 500 ml of LB media.

3. Allow the culture to grow to an optical density of ~0.4.

4. Place all of the culture into 200 ml disposable centrifuge tubes (conical).

5. Centrifuge the cells at 4°C for 5 min. at 2000 rpm.

6. Combine and resuspend the cell pellets in 100 ml of cold 50 mM $CaCl_2$.

7. Aliquot the resuspended cells into six 50 ml conical tubes that have been pre-chilled on ice.

8. Spin at 2000 rpm at 4°C for 5 min.

9. Gently resuspend the cells in 20 ml of cold 50 mM $CaCl_2$ plus 15% glycerol.

10. Aliquot 100 µl of the resuspended cells per tube (enough for 1 transformation).

11. Flash freeze the tubes on dry ice.

12. Store the flash-frozen tubes at -80°C.

To conduct the transformation, follow the procedure below.

1. Remove competent cells (DH5alpha) from a -70°C freezer and place the cells on ice to thaw.

2. Place Eppendorf™ tubes on ice to chill. When thawed, add 200 µl of competent cells to each of the chilled tubes. The number of tubes corresponds to the number of transformations. Refreeze any unused cells in a dry ice/ethanol bath for 5 min., and return to the -70°C freezer.

3. Add all of each ligation reaction (20 µl) to each of the tubes containing 200 µl of cells. Mix gently.

4. As a control to test transformation efficiency, also add 5 µl of 0.1 ng/µl (i.e. 0.5 ng) of control pUC19 DNA provided by BRL to 50 µl of competent cells.

5. Incubate cells on ice for 30 minutes. Gently mix cells after 15 min. of incubation.

6. Heat shock cells at 37°C for 1 minute. Do not shake.

7. Place cells on ice for 2 minutes.

8. Add 800 µl of L.B. medium.

9. Shake at 225 rpm for 1 hour at 37°C to express ampicillin resistance.

10. In the interim, remove the appropriate number of LB-Amp plates out of cold storage, and spread 20 µl of 50 mg/ml X-GAL and 100 µl of 100 mM IPTG onto each plate.

11. After 1 hr. of incubation, pellet cells (except for the tube containing the pUC19 control DNA) in a microcentrifuge by centrifugation for 1 minute. Decant supernatant.

12. Resuspend cell pellets in 100 µl of L.B.

13. Plate 50 µl of each suspension on a L.B.-Amp plate containing X-GAL and IPTG. Plate 10 µl of the 1 ml of pUC19 control transformation.

14. Incubate the plates overnight at 37°C.

E. PCR characterization of inserts

1. With a sterile toothpick, pick a white colony and lightly touch a plate of LB-Amp with it (do not streak) to transfer some cells onto the plate.

2. Immediately dip the same toothpick into an Eppendorf™ tube containing 25 μl of 5 mM Tris-HCl, 0.1 mM EDTA (pH=8.0). Shake the toothpick to suspend cells and then discard the toothpick.

3. With a fresh toothpick, streak out cells that were patched onto the LB-Amp plate. Incubate the plates overnight at 37°C.

4. Boil buffer-suspended cells for 5 min. by placing them in a beaker of boiling water over a Bunsen burner flame.

5. Spin down cellular debris for 1 min. in a microcentrifuge.

6. Transfer 3 μl of supernatant to wells of a microtiter dish for the PCR reaction.

7. For the PCR reaction, prepare a master mix by combining: 20 μl 10 mM dNTP mix; 2.74 μl of Universal primer (1 mg/ml); 2.33 μl of Reverse primer (1.1 mg/ml); 100 μl of 10X Taq polymerase buffer (Boehringer Mannheim); 10 μl of Taq DNA polymerase (5 U/ml, Boehringer Mannheim), and dH_2O to 1 ml (i.e. 864.93 μl).

8. Add 47 μl of the PCR master mix to each well of the microtiter dish containing 3 μl of boiled cell lysate.

9. Mix and cover with mineral oil.

10. Place microtiter dish in a PCR machine, and perform the PCR reaction by first incubating at 95°C for 5' and then performing 30 cycles of 94°C for 10"; 54°C for 30"; and 72°C for 30".

11. Add loading dye to the PCR reactions, and load onto a 2% agarose gel with the 123 bp marker DNA (2 μl).

12. Examine gel to confirm that the bands are of the expected size.

Note that about 220 bp of vector sequence is being amplified in addition to insert sequence, so the size expected for the band on the gel is equal to the DD band size plus 220 bp. Those clones that appear to contain inserts of the correct size can then be sequenced.

F. PCR Sequencing of bacterial colonies

___ For this protocol, one should use fresh colonies (i.e., one or two day old plates).

1. As in the procedure for characterization of inserts of transformants, pick colonies with sterile toothpicks, and resuspend the cells in 25 μl of 5 mM Tris-HCl, 0.1 mM EDTA (pH=8.0). This time, however, cells are picked from plates containing streaked-out colonies from transformation plates, not from the original transformation plates.

2. Boil cells for 5 min., place on ice, and spin down cellular debris for 1 minute in a microcentrifuge.

3. Transfer 10 μl out of supernatant to a fresh Eppendorf™ tube to sequence.

4. To 10 μl of the supernatant, add the following: 1 μl of 1 mM Universal primer (1 pmol); 4 μl of 10X sequencing buffer; 1 μl (10 mCi) of [alpha-^{32}P]-dATP; 1 μl (2 U) of Taq polymerase; and dH_2O to 30 μl (i.e. 13 μl).

5. Add 3 μl of each of the four ddNTP (G, A, T, C) mixes to the wells of a microtiter dish, according to the number of sequencing reactions being performed.

6. Aliquot 7 μl of the sequencing reaction mixture from step 4 into each of the 4 termination tubes containing 3 μl of ddNTP. Mix and overlay with one drop of mineral oil.

7. Run reactions in the PCR machine under the following conditions: an incubation at 95°C for 5', followed by 30 cycles of 95°C for 30"; 60°C for 30"; and 72°C for 1'.

8. When reactions are complete, add 5 μl of stop mix to each well.

9. Heat-denature the samples for 5' at 95°C in a PCR machine, and load 5-6 μl on a 6% acrylamide sequencing gel.

10. Run the gel until the xylene cyanol dye front is approximately 5 cm from the bottom of the gel, so that

the sequence corresponding to the *Hin*dIII site will be near the bottom of the gel.

G. Analysis of DNA sequence from clones of a band

1. Locate the sequence of the *Hin*dIII site in the clones, thus locating the beginning of the insert sequence. Confirm that vector sequences are present.

2. Scan the different clones to see if any match another. Also compare sequences to sequences previously determined directly from acrylamide slices for matches. Pick clones that match previous sequences for mini-prep analysis.

At this stage, there is no need to read and record the sequences in detail, as this can be done when the plasmids from small-scale plasmid preparations ("minipreps") are sequenced. Instead, scan sequences for comparison purposes to determine the clones that should be differentially expressed.

Sequences determined directly from acrylamide slices were obtained using the 5'-Differential Display primers as sequencing primers. Therefore, these sequences are from the 5' end of the bands. The bands cloned in this procedure were inserted into pBluescript non-directionally. Therefore, the sequences obtained from the Universal primer can start from the 5' or 3' end. One can distinguish which end is being sequenced by presence or absence of a long stretch of T residues (complementary to the poly A tail of the 3'-end) immediately after the *Hin*dIII site. The presence of the stretch of T's indicates the 3'-end of the gene is being read, whereas its absence means the 5'-end of the gene is being read. If the 5' end of the gene is being read, then this sequence can be compared directly to the previously derived sequences obtained from the gels. If, however, the 3'-end of the gene is being read, the sequence has to

be "inverted" to make a comparison with the gel-derived sequences. The clone may have to be sequenced from the "other end" with the Reverse primer to obtain the 5'-end sequence so that a direct comparison can be made.

Where sequence information was not directly obtainable from the gels (i.e., the "Unknown" genetags in Table 2, above), steps 1 and 2, above, apply, except that one does not initially know what sequence corresponds to the differentially displayed band. One should look for which sequences match, and pick the sequence that appears the most frequently for mini-prep analysis. This may mean picking more than one band to mini-prep and test by Northern analysis to determine which is the differentially expressed band.

H. Alkaline lysis miniprep procedure

This procedure is adapted from a procedure described in Book 1 of Molecular Cloning by Sambrooke, Fritsch and Maniatis (pp. 1.25-1.28).

1. Prepare overnight cultures of cells containing the plasmid of interest in 5 ml of LB-Amp (5 ml of LB-Amp should contain 5 µl of 100 mg/ml Ampicillin for a final concentration of 100 µg/ml).

2. Transfer 1.5 ml of culture to a fresh Eppendorf™ tube and pellet cells in a microcentrifuge for 1 minute. Transfer another 1.5 ml of the culture to the same tube, and pellet these cells, so that the tube contains the combined pellets from 3 ml of culture.

3. Resuspend pellets in 100 µl of Solution 1. Vortex briefly.

4. Add 200 µl of 1% SDS, 0.2 M NaOH. This solution should be freshly made (just before using) from stocks of 20% SDS and 10 M NaOH. Mix gently by inversion to resuspend cells. Leave on ice for 5 minutes.

5. Add 150 µl of Solution 3. Mix gently by inversion and incubate 5' on ice.

6. Centrifuge the solution in a microcentrifuge for 2-4 min.

7. Transfer the supernatant to a fresh tube and discard the pellet. Phenol/chloroform extract (400 µl) the supernatant, shaking the tube by hand for 20" and then centrifuging the tube for 2 min. Transfer the top (aqueous) layer to a new tube.

8. Add 1 ml of 100% ethanol to the solution and mix.

9. Pellet the DNA by centrifugation in a microcentrifuge for 10'.

10. Remove the supernatant. Add 200 µl of 70% Ethanol to wash the pellet, and centrifuge for 2' in a microcentrifuge.

11. Remove the supernatant by vacuum suction, making certain that all of the EtOH is removed.

12. Dissolve the pellet in 50 µl of TE buffer.

13. Digest 8 µl with HindIII in the presence of 1 µl of DNase-free RNase (concentrations of 500 µg/ml - 3 mg/ml are satisfactory).

14. Run entire digest on a 2% low melting point agarose gel, and confirm the insert is of the right size (using the 123 bp marker). Cut out the band from the gel, and place the band in an Eppendorf™ tube. This band can then be used to prepare a probe to screen Northern Blots.

Example 8

Making Oligonucleotide Probes from Known Genes

When one has identified a putative senescence-related gene or genetag, one can readily verify that the gene or genetag is senescence-related by Northern analyses or *in situ* hybridization, as discussed above. For both of these procedures, however, one requires a probe that is relatively specific, for if the probe hybridizes to a

sequence that is rather abundant in the RNA population of a cell, then the results of any procedure involving probe hybridization to an RNA in that population could be ambiguous. Consequently, there are advantages to first checking the specificity a probe sequence prior to performing a Northern blot or in situ hybridization experiment.

Where the putative senescence-related gene is a known gene, one can readily prepare a variety of synthetic oligonucleotide probes from the known sequence. Typically, such probes are 20 to 60 nucleotides in length, with longer probes preferred for specificity. The specificity of such probes can be conveniently analyzed by Southern hybridization against genomic DNA. If the probe only hybridizes to a few bands (less than 5, and preferably less than 3 bands), then the probe is specific enough for use in verifying that a gene is senescence-related and for use in screens to determine whether a compound affects expression levels of senescence-related genes in senescent cells.

To prepare an oligonucleotide probe from a known gene, one can conveniently label the probe with a radioactive label by a kinase reaction procedure. To prepare a 20 µl kinase reaction, use 600 ng of oligonucleotide (6 µl of a 40 mer oligonucleotide solution at 100 ng/µl), and add the following ingredients: 7 µl of water; 2 µl of 10X One-phor-all™ buffer (Pharmacia); 2 µl of Pharmacia T4 polynucleotide kinase; and 5 µl of gamma-^{32}P-ATP (3000 Ci/mmol). Incubate the reaction mixture at 37°C for 30', and purify the labelled probe over a Pharmacia S-200 Sephacryl™ spin column.

The following Example describes how to prepare probes from genetags of novel genes, and Examples 10 and 11 describe how to perform the Southern analysis to check for the specificity of probe hybridization.

Example 9

Making Probes from EDD-Cloned Bands

When one identifies a genetag of a novel gene as a putative senescence-related gene, then only the genetag sequence is initially available for use in probe design. To perform a rapid analysis of whether the genetag sequence can be used as a probe with a high degree of specificity, one can conveniently prepare probes from the genetag clone (see Example 7) by digesting the plasmid with HindIII (for plasmids prepared as per Example 7), separating the resulting fragments on a low-melting agarose gel (as per the procedure in Example 7), and separating and removing the band from the gel as follows.

1. Melt 10-15 µl of the gel slice at 65°C for 2'.

2. Add 10 µl of 20 µM 5'-primer and 10 µl of 20 µm 3'-primer to the gel slice solution.

3. Heat this solution at 100°C for 10' and then quick-cool on ice.

4. Then, add: 2 µl of BSA (10 mg/ml); 1.5 µl of Klenow enzyme; and 10 µl of a 5X oligonucleotide-labelling buffer containing neither primers nor dCTP. The 5X Labeling Buffer contains 250 mM Tris (pH=8); 25 mM $MgCl_2$; 5 mM beta-mercaptoethanol; 2 mM dATP, dTTP, dGTP; 1 M HEPES (pH=6.6); and 5 µl of alpha-^{32}P-dCTP (3000 Ci/mmol).

5. Incubate this solution at 37°C for 30'.

6. Heat this solution briefly to melt the gel slice if the gel slice has solidified, and then purify the labelled probe over a Pharmacia S-200 spin column.

Example 10

Genomic Digests, Gel Electrophoresis, and Transfer for Southern Analysis

To check the specificity of a probe for use in Northern analyses or *in situ* hybridization, one can hybridize the probe to restriction enzyme-digested genomic DNA. To prepare the genomic DNA, one first isolates (or purchases) genomic human DNA by any of a variety of standard methods and then digests the DNA with restriction enzymes. For best results, the restriction enzyme digestion is conducted with a variety of different restriction enzymes that have 6-base recognition sequences, i.e., *Hin*dIII, *Eco*RI, and *Bam*HI.

Restriction digest conditions are 500 µl of total reaction volume containing 10 µg of human genomic DNA, 50 µl of 10X restriction enzyme buffer, and 5 µl of restriction enzyme, and the reactions are incubated at 37°C for 30 min., at which point, one can add an additional 5 µl of enzyme and let the reaction continue for 6 hrs. The digested DNA is precipitated by adding 50 µl of 3 M NaAcetate and 1 ml of cold 100% ethanol and centrifuging for 10 min. at room temperature at 14K. Aspirate the ethanol, and let the DNA pellet air dry for 20 min., or dry the DNA in a speed-vac centrifuge for 10 min. Resuspend the DNA in 20 µl of water by incubation at 4°C overnight (pellet may require more time to resuspend).

For an 0.8% agarose gel, add 2.4 g of agarose to 300 ml of 0.5X TBE buffer. Add 3 µl of loading dye to each sample, and load the sample into the 0.8% agarose gel. Run the gel at 35 V for 6 hrs. The lower voltage and longer running time tighten up the restriction fragment bands. Take a photograph of the gel after staining with ethidium bromide.

To blot the gel, soak the gel in 0.6 M NaCl and 0.4 M NaOH for 10 minutes at room temperature. The transfer solution is the same as the soak solution. Place the gel upside down on the filter paper wick. Cut a piece of Schleicher and Shuell membrane to size, and place the rough side down onto the gel. Place two pieces of filter paper on top of this membrane, then place a stack of terriwipes on top with a weight pressing the terriwipes down. Cover the buffer chambers with saran wrap and allow the transfer to continue overnight.

After the transfer, soak the membrane in 5X SSC buffer for five minutes; then, blot excess liquid off. Place the membrane in the Stratalinker™ light box (Stratagene) with the DNA side up, and cross link at 1200 kJ (autocrosslink mode). Check the flattened gel to make sure there is no DNA left in the gel.

Example 11

Southern Hybridization and Wash Conditions

A. Hybridization

Prehybridize the blot for 2 hours at 60°C using the following prehybridization solution: 5X SSC (from 20X SSC purchased from Boehringer Mannheim); 1X Denhardt's (50X contains 5 g Ficoll (Type 400, Pharmacia); 5 g polyvinylpyrrolidone; 5 g BSA (Fraction V, Sigma); and water to 500 ml; this stock solution should be distributed into 4 ml Corning tubes and stored at -20°C); 0.1% SDS; 0.05% sodium pyrophosphate (diluted from a 5% stock solution and 150 µg/ml denatured salmon sperm. After the 2 hour prehybridization incubation, remove all of the solution, and replace with hybridization solution, which is the same as prehybridization solution with the addition of 10% dextran sulfate. Boil the probe for several minutes, quench briefly on ice, and then add this solution to the blot soaking in the hybridization

solution. Double bag the blot, and allow the hybridization to continue at 60°C overnight.

B. Wash Conditions

After the hybridization, the probe can be collected and saved in a 15 ml Corning tube at room temperature. Count 5 µl of this probe solution in the scintillation counter. Rinse the blot twice at room temperature in the following solution: 3X SSC, 0.1% SDS, and .05% sodium pyrophosphate. Conduct four 15' washes at 60°C with this solution, which should be pre-heated to 60°C.

C. Analysis

After the Southern blot is probed and washed, the blot is analyzed to determine where the probe is bound on the membrane by PhosphoImager™ analysis (Molecular Dynamics) or autoradiography. Probes with suitable specificity for use in Northern analyses and in situ hybridizations are identified by a hybridization pattern in which the probe has hybridized to only a few (less than 5 and preferably less than 3) bands.

Example 12

Denaturing RNA Agarose Gels for Northern Analysis

When making a formaldehyde gel, always use baked glassware, protective gloves, and DEPC-water. To prepare the gel, add 2 g of agarose to 140 ml of DEPC-H_2O (which in turn is prepared by adding 1 ml of DEPC to 1000 ml of deionized water, resulting in a 0.1% solution of DEPC, heat the resulting solution with stirring in a hood for several hours, and autoclave); boil the resulting solution in a 500 ml flask for approximately three minutes, and then place the solution in a 60°C water bath to equilibrate. De-ionize 65 ml of 37% formaldehyde by adding several grams of mix bed resin (from Bio Rad) to

the formaldehyde in a baked ehrlenmyer flask in a hood, mixing with a stir bar for approximately five minutes, and then filtering away the resin by pouring this mixture through a funnel coated with 3MM circular Whatmann™ paper, after which the formaldehyde is ready to be used.

Next, add 44 ml of 5X running buffer to the agarose/water solution being incubated at 60°C. 5X running buffer is composed of 0.1 M MOPS (pH=7), which is prepared from a 0.5 M stock solution in which the pH has been adjusted using either NaOH or Acetic Acid and stored at room temperature in a bottle wrapped in aluminum foil; 40 mM sodium acetate; and 5 mM EDTA. Then, add 40 ml of the 37% deionized formaldehyde, mix the solution well, and pour the gel in a hood. Pre-run the gel for 5' at 150 volts in the hood; always wear gloves when handling the gel box.

Samples are prepared as follows. One typically loads 20 μg (4.5 μl) of RNA per lane, which is mixed prior to loading with 2 μl of 5X running buffer; 3.5 μl of deionized formaldehyde; and 10 μl of formamide. This mixture is heated for 15' at 65°C and then centrifuged briefly to collect the mixture at the bottom of the tube. Add 2 μl of a 10X loading buffer (which consists of: 50% glycerol; 1 mM EDTA, pH=8; 0.25% bromophenol blue; 0.25% xylene cyanol FF) to each sample. Prepare the lambda-HindIII standards in the same way as above (denatured) using 1 μg of DNA per lane. Load the samples on the gel, and run the gel at 80 volts for 3 to 4 hours in the hood. Stop the gel half-way through the run to shake the gel lightly, circulate buffer, and add new buffer. After the gel has finished, wash the gel 3X in DEPC-H_2O with each wash lasting 10 minutes to remove most of the formaldehyde. Rinse the gel in the gel-casting tray.

Cut off the lambda-HindIII lane with an RNA lane next to it to stain in ethidium bromide for visualization. To perform the ethidium stain, place the gel slice in a gel-

casting tray filled with DEPC-H_2O; add a few drops of 5 mg/ml ethidium bromide to the tray; stain for 20 minutes at room temperature; perform two 10 minute washes in DEPC-H_2O; leave overnight in water in the refrigerator to destain completely; and place the gel next to a ruler on the light box to visualize. Take a photo with the aperture set at 8 and the time set at 0.25 seconds. Take the rest of the gel and blot the gel onto a positively charged nytran membrane, 0.45 µm pore size, "Nytran + Maximum Strength" (Schleicher and Schuell).

Next, one sets up the transfer apparatus; there is no need to pre-wet the membrane. Place the membrane onto the gel; then, place two pieces of 3MM whatman paper on top of the membrane, place a stack of terri towels on top of paper, and place a heavy weight on top of the towels. The transfer buffer is 20X SSC; the transfer should be allowed to continue overnight. Label the membrane with a pencil or ball point pen; wash the membrane in 5X SSC for several minutes; and lightly blot the membrane with filter paper. To link the RNA to the membrane, use the Stratalinker™ (autolink mode, 120 mJ/cm^2) light box. Place the membrane on a piece of filter paper with the RNA side up. under the light.

Example 13

Northern Hybridization and Wash Conditions

Prehybridize the blot for at least 30' at 42°C using hybridization solution composed of 50% formamide; 5X Denhardt's; 0.5% SDS; 5X SSPE or SSC; and 100 µg/ml of salmon sperm DNA. Boil the salmon sperm DNA to denature the DNA before adding it to the hybridization solution. If one is reusing hybridization solution, boil the solution for 2' and quick cool on ice before adding the solution to the blot. After boiling the probe (DD bands), one adds the probe to the blot, double-bags the

blot plus hybridization solution, and incubates the blot at 42°C overnight.

After hybridization, the probe is collected (and can be reused; store at room temperature), and the blot is rinsed twice at room temperature in the a solution composed of 1X SSC and 0.5% SDS. Then, two 30' washes at 65°C are performed using the same (but fresh) wash solution to wash away non-specifically bound probe, leaving only the specifically bound probe.

As noted above, the Northern analysis procedure can be used to confirm that a genetag or probe can specifically identify a senescence-related gene. Alternatively, one can use RT-PCR (Reverse Transcriptase-mediated Polymerase Chain Reaction) for this purpose. After a gene or genetag has been confirmed to be a senescence-related gene for cells in tissue culture, one can confirm that the gene is senescence-related in vivo by an in situ hybridization procedure, such as the procedure described in the Novagen Suresite™ II System manual, supra, using tissues from young and old donors.

Once a probe has been confirmed as identifying specifically a senescence-related gene, the Northern analysis procedure or RT-PCR can be used to identify whether a compound can reverse, partially reverse, or modulate the pattern of expression of senescence-related genes in senescent cells. Once such a compound is identified, one can determine whether the compound has activity in vivo by analyzing tissues from treated animals with an in situ hybridization procedure. The in situ procedure can also be used to identify senescent or young cells in tissues using senescent-related gene probes of the invention that have been confirmed to identify senescence-related genes in vivo.

Example 14

ß-Galactosidase Screen

A. Primary Screen.

Senescent cells are seeded in 96-well plates at 10,000 to 20,000 cells/well in DMEM medium plus 10% Bovine Calf Serum (BCS). In two preferred embodiments, senescent human embryonic lung fibroblasts (IMR90 cells) are used at Passage Doubling Level (PDL) 53, or senescent fibroblast lines derived from human foreskin (BJ cells) are used at PDL 92. Other senescent cells, in the appropriate media, can also be used. After 6 hours, the medium is removed and replaced with DMEM plus 0.5% BCS. After 3 days, the medium is replaced with fresh medium, and the sample or its vehicle is added. In a preferred embodiment, 2 µl of sample dissolved in DMSO (1 µM final concentration), or of DMSO alone, are added to 200 µl of medium. Other volumes, vehicles, and compound concentrations can also be used. In addition, mixtures of compounds, rather than single compounds, can be added to the cells. After 4 days (or other appropriate incubation time), the medium is again removed and the cells are fixed and stained.

To fix the cells, the medium is removed and replaced with phosphate-buffered saline (PBS). This, and all other liquid transfers can be accomplished using a Hamilton Microlab 2000™ pipeting station. Other pipeting stations, or manual pipetting, can also be used. The PBS is then removed and again replaced with PBS. The PBS is removed and replaced with freshly-prepared fixing solution (0.5% gluteraldehyde in PBS). The cells are incubated in this mixture for 2 min. at room temperature. The fixing solution is removed and replaced with PBS. The PBS is replaced with fresh PBS, and the cells are incubated for an additional 10 min. at room temperature. Other methods of fixing the cells can also be employed.

To stain the cells, 100 µl of X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactosidase, at a concentration of

50 mg/ml in dimethylformamide Promega, Madison, WI), is added to 10 ml of staining buffer (40 mM citric acid/Na_2HPO_4 buffer pH=6, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$, in distilled water). The X-gal can be replaced with other substrates for ß-galactosidase in the appropriate buffer. The PBS is removed and replaced with a sufficient volume of staining solution (50 µl are routinely used) to cover the cells. The microtiter plate is then covered, sealed in a humidified container, and placed in an incubator at 37°C overnight. Movement of the plates between the pipetting station and the incubator can be done either manually or with a robot (e.g. Zymark XP (TM)).

Following the overnight incubation, the intensity of staining is measured by, e.g., using a plate reader at a wavelength of 540 nM. Quantitation can also be performed microscopically or with the aid of an image-analysis system. Decreased intensity of the staining in the presence of the test compound indicates reversal of the senescent phenotype. Samples that produce this affect are tested in the secondary assay. The plates can be sealed and stored at 4°C indefinitely.

B. Secondary Screen

Samples that decrease staining in senescent cells are then tested for ability to decrease staining in young cells. In two preferred embodiments, young IMR90 cells are used at a PDL lower than 35, or young BJ cells are used at a PDL lower than 55. Other young cells, in the appropriate media, can also be used. The secondary screen is carried out in the same manner as the screen in senescent cells with two modifications. First, young cells, rather than senescent cells, are used. Second, the staining buffer is adjusted to pH 4 rather than pH 6. A decrease in ß-galactosidase staining in senescent but

not young cells is interpreted as a reversal of the senescent phenotype.

Other high-throughput screens for compounds that alter the expression of specific senescence-related genes include screens for fibronectin, collagen 1 (alpha 1 and 3), and elastin (see Ahmed et al., 1992, *A colorimetric microassay for glycated collagen based on the thiobarbituric acid method*, Clinica Chimica Acta. 212: 133-139; Anderson and Elliot, 1991, *A dye-binding assay for soluble elastin*, Biochem. Soc. Trans. 19:388S; Clark et al., 1992, *Monoclonal antibodies against human fibroblast collagenase and the design of an enzyme-linked immunosorbent assay to measure total collagenase*, Matrix 12: 475-480; Walsh et al., 1992, *Microplate reader-based quantitation of collagens*, Analyt. Biochem. 203: 187-190; and Scutt et al., 1992, *A semiautomated, 96-well plate assay for collagen synthesis*, Analyt. Biochem. 203: 290-294). As with the beta-galactosidase screen described in this Example, one first determines whether a compound can modulate the activity or expression level (protein levels can be determined, for example, by gel analysis or by antibody-based methods) of a senescence-related gene and then determines whether the compound has the modulatory effect on a panel of senescence related genes, preferably using Northern analysis, RT-PCR or in situ hybridization with probes from known senescence-related genes, from known genes that have been determined to be senescence-related by the method of the present invention, and from senescent gene-related genetags from previously unknown genes provided by the present invention.

Example 15

Senescence-related Gene Expression Screen

To determine the effect of a compound on the mRNA levels of known and novel senescence-related genes

according to the method of the present invention, senescent cells are grown in a 10 or 15 cm plate using the same protocol as for the high throughput screens. One plate is incubated with the test compound, and another plate is incubated with the compound test vehicle alone. After four to 20 days of incubation, the cells are lysed in GITC (see the RNA isolation protocol, above), and RNA is prepared. The RNA is analyzed with the senescence-related gene probes of the invention by Northern analysis or by other suitable methods, such as RT-PCR. The results of this analysis will indicate the efficacy of the compound in altering the mRNA expression level on senescence-related genes. The expression levels of at least two, and preferably 3 to 5 to 10 to 20 or more, senescence-related genes will be determined.

Thus, compounds are tested to determine whether the compounds alter the expression of the young- and old-specific senescence-related genes identified by EDD and in the scientific literature. If a compound has the effect of complete reversal to a young pattern of gene expression, then the compound impacts a single common mechanism driving cell senescence. Reversal of defined groups of genes indicates that several mechanisms are operating in senescence and that different mechanisms can affect different panels of genes. A compound may also act to modulate the activity of an individual gene, suggesting the absence of a common mechanism.

An alternative screen for compounds that alter the expression of senescence-related gene involves the use of a genetic construct comprising a promoter of a senescence-related gene positioned for expression of a coding sequence from a reporter gene, such as an alkaline phosphatase gene, the expression of which can be efficiently and readily monitored. Such a construct would be used to generate stable transfectants in very young cells, such as dermal fibroblasts, and then the

cells could be used at any stage up to and including senescence to identify agents that up or down-regulate the expression of the reporter gene.

The foregoing examples describe various aspects of the invention and how the methods of the invention can be practiced. The examples are not intended to provide an exhaustive description of the many different embodiments of the invention. All publications and patent applications cited above are hereby incorporated herein by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Thus, although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Maarten H.K. Linskens, et al.

(ii) TITLE OF INVENTION: METHODS AND REAGENTS FOR THE IDENTIFICATION AND REGULATION OF SENESCENCE-RELATED GENES

(iii) NUMBER OF SEQUENCES: 130

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(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 08/332,420
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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCGCAAGCTT TTTTTTTTTT CT 22

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCGCAAGCTT TTTTTTTTTT CC 22

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCGCAAGCTT TTTTTTTTTT CG 22

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCGCAAGCTT TTTTTTTTTT GT 22

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: :

GCGCAAGCTT TTTTTTTTTT GG 22

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCGCAAGCTT TTTTTTTTTT GA 22

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCGCAAGCTT TTTTTTTTTT AT 22

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCGCAAGCTT TTTTTTTTTT AC 22

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GCGCAAGCTT TTTTTTTTTT AG 22

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCGCAAGCTT TTTTTTTTTT AA 22

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCGCAAGCTT TTTTTTTTTT CA 22

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GCGCAAGCTT TTTTTTTTTT GC 22

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CGGGAAGCTT ATCGACTCCA AG 22

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CGGGAAGCTT TAGCTAGCAT GG 22

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CGGGAAGCTT GCTAAGACTA GC 22

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CGGGAAGCTT TGCAGTGTGT GA 22

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CGGGAAGCTT GTGACCATTG CA 22

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CGGGAAGCTT GTCTGCTAGG TA 22

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CGGGAAGCTT GCATGGTAGT CT 22

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CGGGAAGCTT GTGTTGCACC AT 22

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CGGGAAGCTT AGACGCTAGT GT 22

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CGGGAAGCTT TAGCTAGCAG AC 22

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CGGGAAGCTT CATGATGCTA CC 22

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CGGGAAGCTT ACTCCATGAC TC 22

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CGGGAAGCTT ATTACAACGA GG 22

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CGGGAAGCTT ATTGGATTGG TC 22

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CGGGAAGCTT ATCTTTCTAC CC 22

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CGGGAAGCTT ATTTTTGGCT CC 22

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CGGGAAGCTT TATCGATACA GG 22

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

CGGGAAGCTT TATGGTAAAG GG 22

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CGGGAAGCTT TATCGGTCAT AG 22

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

CGGGAAGCTT TAGGTACTAA GG 22

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

CATTTATTCA TTCATTGAGA CACTCAA 27

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 112
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

ACAGAAAGGC CACTCAGGAT GTCCTTTGTG TCCATTGATG TCATTCAGCA GTCAGTCCCC 60
CAATAATCCT TAAACTAGCT AAAACCAAAG GTAGTCNTTA GAAGATCTGC TT 112

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 110
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTGAGTAGTT ACTGGAACCT TGACATTGCC TTTTAATGAG GTACTTCCAA AAAAAGGACC 60
CCTAACAATG GCATAATAGT GAGGTCTCTC TGTGCGTGTA CATAATATA 110

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

CAAAGATAAG AAACCAAGGA AGAAAGCAA 30

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

CTGACGCCAN CCGCATACNC CGCANCCACA 30

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

AGATAAAGCA ATTAGAAGAT GCATTAAAAG ATGTGCAGAA GAGGATGTAT GAGTCAGAAG 60

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 86
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

ATAATAAAAC TCTTCATTTT GCGAATTATA GAAGCTACTT TTTATAAAGC CATATTTTTT 60
TAGGGAAACT AAGGAGTGAC ATAGAA 86

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

AACTGCATTT TGATGTTATC GCTTATGTTT AATAGTTAAT TCC 43

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

CTATTGCCTC TCCTCCTGCA GAGACCATG 29

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

GAGAAGAAAG GAAAGAAAGG NCACAGAGAT GGAAGGCCA 40

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

GTTTCTGAAT TACATGAATT GTTGCAGAGC AAAGAAACTT ATGGAAATCT TTCCATTTAT 60

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

GTAGGCTTCT ATATTGCATT TAACTTG 27

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 72
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

AATGAGGTAG AAGTAGAAAG GAAGAAAAAC TCAAAGAATT CTAAAAGGAT TCATAGCAAC 60
ATAATGTGTC CC 72

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

TCTCACATTC AGTCATACCC TAATGATCCC AGAAAGATAA TCAT 44

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

AGAAGCCCCA GCAAGATTTA TTCCTTTTTG CTTCTTCATA ACCATGAAGC CATTGAAC 60

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

CTACCTCCCA CATTAATTTT CATATGT 27

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

AGGGCACAGC ACCAGATGAA TTGTTGTATA T 31

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

AAATTAGCTT TCATCACAGA TTTAGGAAACT TGTCT 35

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

AAACTACTGA ACNGTTACCT AGGTTAACAAC CCTGGTTGAG TATTTGC 47

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

TTGNATATTG NATTTGTAGT AATATTCCAAA AGAATGTAAA TAGG 44

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

AAATTGTATA TTGTATTTGT AGTAATATTCC AAAAGAATGT 40

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

TATGAATNTC ACATTTGAAT TCTTCGATCTC TAA 33

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

TATGTATAAA AGCATATGTG CTACTCATCTT TGCTCAC 37

(2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

AATGTCTAAT TTTCTTTCCG ACACATTTACC AAA 33

(2) INFORMATION FOR SEQ ID NO: 57:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 57:

ACAACAGCAA ACAAAAAGGT GAAGTCTAAAT GAAGTGCACA 40

(2) INFORMATION FOR SEQ ID NO: 58:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 58:

AAAAGAATTG GCAGTTACAT TCATACTTT 29

(2) INFORMATION FOR SEQ ID NO: 59:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 59:

AAGAATGTGC ATTCCAGTGC CATAGATAGT ATATTGAA 38

(2) INFORMATION FOR SEQ ID NO: 60:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 60:

TTGCTACGGA CTTACGAAAG GACAAAGCGA AGAGCTG 37

(2) INFORMATION FOR SEQ ID NO: 61:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 51
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 61:

AAATAATTTA TTCATTGCAG ATACTTTTTA GGTTGCATTT TATTCATTTC C 51

(2) INFORMATION FOR SEQ ID NO: 62:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 62:

AGATGATGAT GTTAACCCAT TCCAGTACAG TATTCTTTT 39

(2) INFORMATION FOR SEQ ID NO: 63:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 63:

AGTATAGTGA ATGANTATGC CTTCCTACTG CAG 33

(2) INFORMATION FOR SEQ ID NO: 64:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 64:

AGAAATATAA AGATTTTNAT ACCTGCCACA TGG 33

(2) INFORMATION FOR SEQ ID NO: 65:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 65:

GAAGANATTA TGTTGTGANC NGGAGTNACA CAAA 34

(2) INFORMATION FOR SEQ ID NO: 66:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 66:

AGGGGCACAA GAGTTTGCGG TTATTGAATC CTGAGANAA 39

(2) INFORMATION FOR SEQ ID NO: 67:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 67:

GTTGAAGAGA CAGAGACAAG TAATTTGC 28

(2) INFORMATION FOR SEQ ID NO: 68:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 68:

CCGTGAATAC CCNTTTCTCG ACCAAAGA 28

(2) INFORMATION FOR SEQ ID NO: 69:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 69:

ATGGAGTTGT GGATGAAAGC CATGTTAGNT G 31

(2) INFORMATION FOR SEQ ID NO: 70:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 70:

GATCATATAA ACANNNCCGA GTTCTACCTC AGAGTCG 37

(2) INFORMATION FOR SEQ ID NO: 71:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 71:

CCGTTTACCT TCACGTGGCC ATTCTCCTGT CCGTTCGCTT 40

(2) INFORMATION FOR SEQ ID NO: 72:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 72:

AGGAACAATA TTCACTACTC CAGGAGGAAA CCCTGCCTCT 40

(2) INFORMATION FOR SEQ ID NO: 73:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 73:

CCGAGAGATG CTGTAGCGAC CATTTTTCTC CACGTGGTAT 40

(2) INFORMATION FOR SEQ ID NO: 74:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 74:

CCAGGTATTT CTGGACTAAG TCCACATCTT GCTCTTGTGT 40

(2) INFORMATION FOR SEQ ID NO: 75:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 75:

ATCAGCACCT TTGGGACCAG CATCACCTCT GTCACCCTTA 40

(2) INFORMATION FOR SEQ ID NO: 76:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 76:

AAGGTTACTG CAAGCAGCAA CAAAGTCCGC GTATCCACAA 40

(2) INFORMATION FOR SEQ ID NO: 77:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 77:

CGAGAAGTAG CCAGCTCCCC TTTTGCACAA AGCTCATCAT 40

(2) INFORMATION FOR SEQ ID NO: 78:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 78:

CCTGGATAAA AGACTCCTCC AGGAACTCCA CCAGGAATGG 40

(2) INFORMATION FOR SEQ ID NO: 79:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 79:

AAGAAAGGAT CCTCCTCCTC CACCAGCGCC CCTGTGCTGT 40

(2) INFORMATION FOR SEQ ID NO: 80:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 80:

GAGGAGTATT CAGAACTGGT TTCACACCGA AGGACTAGTT 40

(2) INFORMATION FOR SEQ ID NO: 81:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 81:

CTCGTCGGTG AGGTCACACT CTGTGTCTGT TGTGTAAAAC 40

(2) INFORMATION FOR SEQ ID NO: 82:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 82:

CTGGTGCTCC GGTCTCGAAT TTTGGCGAAG TGCTTCTGCA 40

(2) INFORMATION FOR SEQ ID NO: 83:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 83:

CTCCATATTG ATAGGCGTGC TCTATTGCTC TAGGGCTGTT 40

(2) INFORMATION FOR SEQ ID NO: 84:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 84:

TCTTGAATCC CATAGCTGCT TGAATCTGCT GCTGGGTTTC

(2) INFORMATION FOR SEQ ID NO: 85:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 85:

ATTGAGGGCA AAGAGTGTGT TTGCCACACA AAGATCCTCC 40

(2) INFORMATION FOR SEQ ID NO: 86:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 86:

TTGCTTCTGA GCACAGGGCG CAGCCATGAC TGATGTTGCT G 41

(2) INFORMATION FOR SEQ ID NO: 87:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 87:

ATCTGTGGGC ATGGTACGTT TGCTGAAGGA CAGTGGCAGA 40

(2) INFORMATION FOR SEQ ID NO: 88:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 215
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 88:

GCGGCGGCCA TGGCGGGACA GGAGGATCCG GTGCAGCGGG AGATTCACCA 50
GGACTGGGCT AACCGGGAGT ACATTGAGAT AATCACCAGC AGCATCAAGA 100
AAATCGCAGA CTTTCTCAAC TCGTTCGATA TGTCTTGTCG TTCAAGACTT 150
GCAACACTAA ACGAGAAATT GACAGCCCTT GAACGGAGAA TAGAGTACAT 200
TGAAGCTCGG GTGAC 215

(2) INFORMATION FOR SEQ ID NO: 89:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1434
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 89:

CCAGCAATCT ATCATGGATC CTAATCAGAA CGTGAAATGC AAGATAGTTG 50
TGGTGGGAGA CAGTCAGTGT GGAAAAACTG CGCTGCTCCA TGTCTTCGCC 100
AAGGACTGCT TCCCCGAGAA TTACGTTCCT ACAGTGTTTG AGAATTACAC 150
GGCCAGTTTN GAAATCGACA CACAAAGAAT AGAGTTGAGC CTGTGGGACA 200
CTTCGGGTTC TCCTTACTAT GACAATGTCC GCCCCCTCTC TTACCCTGAT 250
TCGGATGCTG TGCTGATTTG CTTTGACATC AGTAGACCAG AGACCCTGGA 300
CAGTGTCCTC AAAAAGTGGA AAGGTGAAAT CCAGGAATTT TGTCCCAAAT 350
ACCAAAATGC TCTTGGTCGG CTGCAAGTCT GATCTGCGGA CAGATGTTAG 400
TACATTAGTA GAGCTCTCCA ATCACAGGCA GACGCCAGTG TCCTATGACC 450
AGGGGGCAAA TATGGCCAAA CAGATTGGAG CAGCTACTTA TATCGAATGC 500
TCAGCTTTAC AGTCGGAAAA TAGCGTCAGA GACATTTTTC ACGTTGCCAC 550

```
CTTGGCATGT GTAAATAAGA CAAATAAAAA CGTTAAGCGG AACAAATCAC    600
AGAGAGCCAC AAAGCGGATT TCACACATGC CTAGCAGACC AGAACTCTCG    650
GCRGTTGCTA CGGACTTACG AAAGGACAAA GCGAAGAGCT GCACTGTGAT    700
GTGAATCTTT CATTATCTTT AATGAAGACA AAGGAATCTA GTGTAAAAAA    750
CAACAGCAAA CAAAAAGGTG AAGTCTAAAT GAAGTGCACA GCCAAAGTCA    800
TGTATACCAG AGGCTTAGGA GGCGTTTGAG AGGRTACTCA TCTTTTTGGG    850
AATCCTGACC TTAGGTTCGG CATGTAGACC AAGTGATGAG AAGTGAATAC    900
ATGGAAGAGT TTTTAAGTGT GACTTGAAAA ATATGCCAAA AAATGAGAGA    950
TACAAATGAG CTAGAGGAAG ATGAGGGGGG ATGCGAGTAC CTCCAAGAAG   1000
AAAAATCACA CTCTGAATGG TGCTTGCATT TTGAGGTTTN NNNNCNNNNG   1050
GGGTATAATC TATCATGGAT CTCCACTTGG ATTAATTTTA AATGTTTAAT   1100
CTCCTTTACA AAAAGTATAC GTTAATATAC CGTCCTCAAG GGGGACTGGC   1150
ACTGTGACCT AGCATTAGTT TCTAGAGGAT GTGATCTAAT TCTTCTAGCT   1200
CATCATAAAA AGGAATTGTA TCAGGACCCA TGGGATATAT CCAGAGGCAA   1250
CTTATGAGGC TTGGAATCTG GCTTCCTGAA GATAGCTGAG TAGGATGGTC   1300
TAAGGAAAGC CTTGGAATCT TGCAAGATTG GTGGACCAGC ACTACAAAGA   1350
TCGCATAGAT CAAATAGGAA AAAAATGTCG ATTTTTATTC AGTCTGATGG   1400
TTCTGTTCTC ATGGTGGATG GTCATAAAAA GTGG                    1434
```

(2) INFORMATION FOR SEQ ID NO: 90:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 265
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 90:

```
GGCACGAGCG GAATTGGACT TGGGAGGCGC GGTGAGGAGT CAGGCTTAAA     50
ACTTGTTGGA GGGGAGTAAC CAGCCTGCTC CTCTCGCTCT CCTCCTCGTC    100
TGCGCCGCGT TTCAGAGGTT GCCCATCAGC CTTGTGATTT ATTTTTATAT    150
CTGCTTTTTA TAAAGAGAGA AATATATATA TATATATATA TATTTTTTTT    200
TTCTTCTTAA GAGAAAATTC CTGTTCCAAG AGAAAATAAG GCAACATCAA    250
TGAAGGAGAG AAGAG                                          265
```

(2) INFORMATION FOR SEQ ID NO: 91:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 223
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 91:

```
GATTGATGCA GCATTATGCT TTGGGCAGTA TTACAAAATA GCTGGCGAGT     50
KCTTTCTGTA TTTAAATATT GTAAAAAGAA AATAAGTTAT AACTGTTATA    100
AAGCAGAACT TTTGTTGCAT TTTTTAAACT GTTGAAGTCA CTGTGTATGT    150
TTGTTTGGTC AATGTTTCCG CAGTATTTAT TAAAACATAC TTTTTTTTTT    200
CTTCAAATAA AAAAGTAACC ATG                                 223
```

(2) INFORMATION FOR SEQ ID NO: 92:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 65
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 92:

TCCTCTCGCT CTCCTCCTCG TCTGCGCCGC TTTCAGAGAG AAAATTCCTG 50
TTCCAAGAGA AAATA 65

(2) INFORMATION FOR SEQ ID NO: 93:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 150
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 93:

ACCATGGAAA GTATAGTGAA TGAATATGCC TTCCTACTGC AGCAAAACTC 50
AAAAAAGCCC ATGACAAATG AGAAACAAAA TTCCATTTTG GCCAACATTA 100
TTCTGAGTTG TCTAAAGCCC AACTCCAAGT TAATTCAACC ACTTACCACG 150

(2) INFORMATION FOR SEQ ID NO: 94:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 108
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 94:

GTTTTTTTTG AGTTTAACAC AGATTTTATT GCCCTATAGA CAGTTATGAT 50
GTGACCAGTG GATATCAATG AAACTTCTTA ATTATTTGAG TCTGAAAATG 100
CATATTTA 108

(2) INFORMATION FOR SEQ ID NO: 95:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 107
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 95:

ACAAACCACA GTATTTCCAC TTTAAATATA GAACTGGTAA ACAGCACTAT 50
CCTTAAACTA AAATCGGTGA CTCGGTCATC AAGAAGGTTT TTGCCCGCCC 100
GTGGATC 107

(2) INFORMATION FOR SEQ ID NO: 96:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 83
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 96:

TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT GAAAGAATAG GTTTAATTTA 50
TTAGTTGCTC TTTAGCAAAG GCTATATAGA ACA 83

(2) INFORMATION FOR SEQ ID NO: 97:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 87
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 97:

GCTAAACCAA ACCAACTCCT CTGCTTTGTC CCTTAGGTGG AAAAGAGAGG 50
TAGTTTAGAA CTCTCTGCAT AGGGGTGGGA ATTAATC 87

(2) INFORMATION FOR SEQ ID NO: 98:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 67
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 98:

TGTTATTATT AGCTTTCCAT GTGGCAGGTA TTAAAATCTT TATATTTCTG 50
AAAAATACTC TTCTCTT 67

(2) INFORMATION FOR SEQ ID NO: 99:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 103
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 99:

GATGGGGACG TCCTGATTTA CCAGATCAAA GTATGGTAAG GCTGTAGATA 50
GCACACTAGT TTTCTCAGGA TTCAATAACC GCAAACTCTT GGTGCCCCTA 100
TTG 103

(2) INFORMATION FOR SEQ ID NO: 100:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 173
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 100:

TTTTTTTTTT TTTTTTTTTT TTAGGAAAGA AAGAATTCTT TTATTCCCAC 50
ATGACAGCCC AATTTTTTAA AATGGTTATC TTAAGTCAGG CCAGTTTTAT 100
TTTATTGACC ATGTATATAT AACATCAGAT ATTTCTAAGA AAGAGAAGAG 150
AACCTGATTG ATGTCTCTCA TGT 173

(2) INFORMATION FOR SEQ ID NO: 101:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 253
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 101:

```
CCCACCGGGG CCATGGCGTC CGCCGACATC GTCGTGGGCG GGGTGGCCCA    50
CGGGCGGCCC TACCTCCAGG ATTATTTTAC AAATGCAAAT AGAGAGTTGA   100
AAAAAGATGC TCAGCAAGAT TACCATCTAG AATATGCCAT GGAAAATAGC   150
ACACACACAA TAATTGAATT TACCAGAGAG CTGCATACAT GTGACATAAA   200
TGACAAGAGT ATAACGGATA GCACTGTGAG AGTGATCTGG GCCTACCACC   250
ATG                                                      253
```

(2) INFORMATION FOR SEQ ID NO: 102:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 261
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 102:

```
GTTTATTAAT ATGATCCAAA CAGATTTAGC TTAAGAGATG AGGACAGGAC    50
ATAAAACAAG ATTTGGCTGA GCTATATAAA GAAAACCAAA AGAGTGACAA   100
GATTTTCAAT TGGTGACAAT TTAAAGTCAT TTATTAACTA ATATAAAAAT   150
AAATCTACAA TAGAACCATG ACTTAAACTG AGCCAGTATG TGGTACATGT   200
GTCACCCAAA GTCAGCAAGG AACCATTCAG AACTTACACC CTCCTGGACT   250
GGCTCACACG G                                             261
```

(2) INFORMATION FOR SEQ ID NO: 103:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 120
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 103:

```
TTTTTTTTTT TTTTAAACTT AAAAATCAGT TTATCTTAAA ATTTTTTTGA    50
ATTAGAAGAA ATTGATTTCA CATGAAAATA TAACATTCTG AAGGTAAGTA   100
TTTTAAACAT TACAACTGTT                                    120
```

(2) INFORMATION FOR SEQ ID NO: 104:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 129
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 104:

```
TGAAAATGCC AAAAATAGTG AACTTAACCT TGTTGGCTAT GATCTGCTAC    50
ACCAGCCAAC ATTATTGTGC CTTTGCATTT CACACCAAAA GTTCCAAATG   100
GGTATTTTTT GATGATGCAA ATGTGAAAG                          129
```

(2) INFORMATION FOR SEQ ID NO: 105:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 214
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 105:

TTTTTTTTTT TTTTTTTTTT TAGGGGGACA AATTTAATTT TTATTCAACT 50
GTAGCATATG ACAAATGAAT TATTATATCT CCCTGATTTT CTATTTTTCA 100
CGGCACGCCA AGACTATTCA AAGGGAATTT CTAAAATATC TAAGATATTG 150
ATCATAAAAT TATATATGTG TATACACATA TACACATACA TATGTATAGT 200
GATTAACAAC TTTA 214

(2) INFORMATION FOR SEQ ID NO: 106:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 214
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 106:

CATGTTTGTC AGGGAGATGC CTGTATATTT TGTGCATTGA AGACGATATT 50
TGCACAGTTC CAACACAGTC GAGAAAAAGC ACTTCCCTCA GATAACATAA 100
GGCATGCTCT TGCAGAAAGT TTCAAAGATG AGCAGCGATT TCAACTTGGC 150
CTTATGGATG ATGCTGCGGA GTGCTTTGAA AATATGTTGG AGAGGATTCA 200
TTTTCACATA GTGC 214

(2) INFORMATION FOR SEQ ID NO: 107:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 264
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 107:

TAAAAATACT ATCAACATTT AATGTTTCTC TCATAGGTTT CCAACCTCGG 50
TTCCGGCTTT TACTGCTGCT ATCACAGCTG TTTCCTCTAT CCCTAGAATC 100
TTGGCTGCTG TTCTGTGTCA TATCCAGTGC CATTGTCACT CTTAACTTTG 150
CCAGTTATTT TCTCTCCTGG AGCAAGAATC TGGGATTTAC TTGAACTTAT 200
TATTTGTGCA GAAGCTMGAC TYTGAGGTAC AACTCGGTCR TGTTTATATG 250
ATCCTTTTCC TTGA 264

(2) INFORMATION FOR SEQ ID NO: 108:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 139
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 108:

GCGCGCCGGC AGCTGCAGGC TCAGGCCATC TCCAGTCTTC CTGCCGCGCC 50
CGCCTCTCCT CCTGGGATTC TCCTCCTCCT CCTGGACTTC CCCGCAGCTG 100
CCCACGCCTC GGCGGCCGCC AGTGCTCCTG GAGTGCAGA 139

(2) INFORMATION FOR SEQ ID NO: 109:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 166

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 109:

CTTTTGCGTC GCCAGCCGAG CCACATCGCT CAGACACCAT GGGGAAGGTG 50
AAGGTCGGAG TCAACGGATT TGGTCGTATT GGGCGCCTGG TCACCAGGCT 100
GCTTTTAACT CTGGTAAAGT GGATATTGTT GCCATCAATG ACCCCTTCAT 150
TGACCTCAAC TACATN 166

(2) INFORMATION FOR SEQ ID NO: 110:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 196
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 110:

TTTTTTGGTT GAGCACAGGG TACTTTATTG ATGGTACATG ACAAGGTCGG 50
CTCCCTAGGC CCCTCCCCTC TTCAAGGGGT CTACATGGCA ACTGTGAGGA 100
GGGGAGATTC AGTGTGGTGG GGGACTGAGT GTGGCAGGGA CTCCCCAGCA 150
GTGAGGGTCT CTCTCTTCCT CTTGTGCTCT TGCTGGGGCT GGTGGT 196

(2) INFORMATION FOR SEQ ID NO: 111:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 148
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 111:

CTGCCAATTC TTGCTCAATT CTAAGGCAGA TAGACTGTGT GAGTTCAAAA 50
GATATCCTCT GAAAAGCATC AAAATCTTCC ACTGTGAACA CATGGGTCTC 100
TGCAGGAGGA GAGGCAATAG CTTCCAATTC TGAGCGAACG GCATCCTC 148

(2) INFORMATION FOR SEQ ID NO: 112:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 88
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 112:

GCAGGGGCTG AAATAACCCA GATGCCCCCA CCCTGCCACA TACTAGATGC 50
AGCCCATAGT TGGCCCCCCT AGCTTCAGCA GTCACTAT 88

(2) INFORMATION FOR SEQ ID NO: 113:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 189
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 113:

```
CTGAAATGCA GCTCCCTGTC CAAGTGCCTT GGAGAACTCA CAGCAGCACG    50
CCTTAATCAA AGGTTTTACC AGCCCTTGGA CACTATGGGA GGAGGGCCAA   100
GAGTACACCA ATTTGTTAAA AGCAAGAAAC CACAGTGTCT CTTCACTAGT   150
CATTTAGAAC ATGTTATCAT CCAAGACTAC TCTACCCTG               189
```

(2) INFORMATION FOR SEQ ID NO: 114:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 151
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 114:

```
CCTCCGCTTA CAGCTCGCTG CCGCCGTCCT GCCCCGCGCC CCCAGGAGAC    50
CTGGACCAGA CCACGATGTG GAAACGCTGG CTCGCGCTCG CGCTCGCGCT   100
GGTGGCGGTC GCCTGGTCCC GCCGAGGAAG ACTAAGACAA ATCAAGATCT   150
T                                                        151
```

(2) INFORMATION FOR SEQ ID NO: 115:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 146
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 115:

```
TTTTTTTTYT TTWWTTTYTT TATTCATCAA TAGTATCCGA AAAGGAAGAA    50
TCAGGAGTTA CAAAAACAAG TTAAATGCAA TATAGAAGCC TACTAAATAC   100
AAATACAAGT CACAAACACA TATGCAAGAG AAACTTGTTT AGATTG       146
```

(2) INFORMATION FOR SEQ ID NO: 116:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 145
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 116:

```
CCGGGCCGGG GAGGCGCGCT CGCTCCGCGC TCCCTTCGCT CGCTCGTTTC    50
CTCCTCCCTC GGCAGCCGCG GCGGCAGCAG GAGAAGGCGG CGGCGGCGGC   100
TAGGGATCAG ACATGGCGGC GGATCTGAAC CTGGAGTGAT CTCCT        145
```

(2) INFORMATION FOR SEQ ID NO: 117:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 216
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 117:

```
TTTTTTTTTT TTTTTTTTAA GAACATCAAC ATTTATTTAA CATGATAAAA    50
```

AAAGAAATGA GATATGAACA TTTGCATTTA AACAATAGTA AGTAGCCTTT 100
AATACATTAC ATGTGCTCAT TGTATAATAT ATACACAATG AACATAATTA 150
CATTTGTACA CAAACTAAGT ACCGGATTTG GAAACCTGCT TATTGCTGTA 200
CACATGTATT CCAATG 216

(2) INFORMATION FOR SEQ ID NO: 118:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 191
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 118:

CACCACGATC AAAAGGGACA AGCATCAAGC ACGCAGCAAT GCAGCTCAAA 50
ACGCTTAGCC TAGCCACACC CCCACGGGAA ACAGCAGTGA TTAACCTTTA 100
GCAATAAACG AAAGTTTAAC TAAGCTATAC TAACCCCAGG TTGGTCAATT 150
TCGTGCCAGC CACCGCGTCA CACGATTACC AAGTCATAGA G 191

(2) INFORMATION FOR SEQ ID NO: 119:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 226
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 119:

TTTTTTTTTT TTTTTTTTTK YTYWTTTYYT TGTTTTTTTG ATTGTTTTGG 50
ATCTCTGGTT TAATTAGCAC TCTATGGTTG GGAATGTTAT TGGTTTCTTT 100
AGTTGGTGCA TTTTCAGATG TAATCTTGTC CACTCTTYTC ACAGGTTCTG 150
TCTGTACTAG GGCAGCATCT AACATGGCTT TCATCCACAA CTCCATTTCC 200
TTTCCTGTAT CAGTGCAGAA AAAAGG 226

(2) INFORMATION FOR SEQ ID NO: 120:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 196
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 120:

CGGATCTGAA CCTGGAGTGG ATCTCCCTGC CCCGGTCCTG GACTTACGGG 50
ATCACCAGGG GCGGCCGAGT CTTCTTCATC AACGAGGAGG CCAAGAGCAC 100
CACCTGGCTG CACCCCGTCA CCGGCGAGGC GGTGGTCACC GGACACCGCG 150
GCAGAGCACA GATTTGCCTA CTGGCTGGGA AGAAGCATAT ACTTTT 196

(2) INFORMATION FOR SEQ ID NO: 121:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 205
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 121:

AGATGGTCCA GCTGCCAGGA CTACTTTGGC AGGCAGCGTG CTACAGGACG 50
AAAATGTAAG AGAAGTCTAT TAAGGCTGGA CAGCCCAGGG TTATTTATAC 100
TCTCTCAGCC CCAAGTCCCC CGGACTAAAG ACCTAAAGGC TGATTGACTC 150
ATTCCTGATT GATTTAATGG AAAGTCTCCC ACCCCATCAT CATTTGCCAG 200
AGTAC 205

(2) INFORMATION FOR SEQ ID NO: 122:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 199
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 122:

CAAAACTCAG CAGTGCTTCT GGTGCTGGTG ATCAGTGCTT CTGCAACCCA 50
TGAGGCGGAG CAGAATGACT CTGTGAGCCC CAGGAAATCC CGAGTGGCGG 100
CTCAAAACTC AGCTGAAGTG GTTCGTTGCC TCAACAGTGC TCTACAGGTC 150
GGCTGCGGGG CTTTTGCATG CCTGGAAAAC TCCACCTGTG ACACAGATG 199

(2) INFORMATION FOR SEQ ID NO: 123:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 123:

TTAGATGCAA GAAGATGCAG GCTCAAAGTC TGGTTGGACA GCCAGGCTCA 50
AGCAATTTGG TAAATGTGTC GGAAAGAAAA TTAGACATTG GAGGATCAAG 100
ACCATAAGAC ACTAGCTCAT TAGAGATCAA GAATTCAAAT GTGACATTCA 150
TATTCGTCC 159

(2) INFORMATION FOR SEQ ID NO: 124:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 124:

ATTTTAAAAG AATACTGTAC TGGAATGGGT TAACATCATC ATCTTTGGCA 50
TCCT 54

(2) INFORMATION FOR SEQ ID NO: 125:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 196
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 125:

GACATTCGCC CTGATATAAA AGATGATATA TATGACCCCA CCTACAAGGA 50
TAAGGAAGGC CCAAGCCCCA AGGTTGAATA TGTCTGGAGA AACATCATCC 100

TTATGTCTCT GCTACACTTG GGAGCCCTGT ATGGGATCAC TTTGATTCCT 150
ACCTGCAAGT TCTACACCTG GCTTTGGGGG GTATTCTACT ATTTTG 196

(2) INFORMATION FOR SEQ ID NO: 126:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 184
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 126:

TCGCACTTTG CCCCTGCTTG GCAGCGGATA AAAGGGGGCT GAGGAAATAC 50
CGGACACGGT CACCCGTTGC CAGCTCTAGC CTTTAAATTC CCGGCTCGGG 100
GACCTCCACG CACCGCGGCT AGCGCCGACA ACCAGCTAGC GTGCAAGGCG 150
CCGCGGCTCA GCGCGTACCG GCGGGCTTCG AAAC 184

(2) INFORMATION FOR SEQ ID NO: 127:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 217
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 127:

TTTTTTAAAT TACAACACTT TATTGCAGCA TCGGCAAAGG TCAGATTTCT 50
GAAGCTGGTG AAGATTGGGC AGCATTTCCA TGTGAAATGT TACAACTTTA 100
CAAGTTTTGT TTTTTATTTA AATCTACATG CAGAAACTGA AACATGGTAA 150
AAGAAAAAAT GCAAAATAGC TAGAAAAAAA GATGTAATCA AGTTGTCGCA 200
TACAGATGTG CTCTCCG 217

(2) INFORMATION FOR SEQ ID NO: 128:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 174
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 128:

CTTGTCTGAC CTAGAAGCTG AGAAACTGGT GATGTTCCAG AGGAGGTACT 50
ACAAACCTGG CTTGCTGATG ATGTGCTTCA TCCTGCCCAC GCTTGTGCCC 100
TGGTATTTCT GGGGTGAAAC TTTTCAAAAC AGTGTGTTCG TTGCCACTTT 150
CTTGCGATAT GCTGTGGTGC TTAA 174

(2) INFORMATION FOR SEQ ID NO: 129:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 234
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 129:

TTTTTTCAGA TTCACTTCAC TTTTATTATG AACAAACACA ATCTCAGATT 50
AGTACAATTA GCTTCAGAGT TGATATTAAT AGAAATTATT CCAAAATTAT 100

```
TCTTGTCACA AGTAACTACT ATATCCCACA TAAAAAGGGA AAAAATCCCA        150
CCCAATCACA GAAAAGGCAT CCTCTGTATG TTTCCGTGGC AATGCGTTGT        200
TTATGTATTC TCAAATTTTG TCTGGCTAGT TATC                         234
```

(2) INFORMATION FOR SEQ ID NO: 130:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 261
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 130:

```
TTGGTGAGGG TGGGGAGGAG TGCTTCGAAG GGAGAAGCCC CAGCAAGATT         50
TATTCCTTTT TGCTTCTTCT TCTCCCTGTC CCTGCCATAA CCATGAAGCC        100
TTGAACAAAC CACCCAAATC TCAGGATCTT AGTGTTTTCT CTGTAAATTG        150
TAATATGAAC TTATAAAGAT CCTCCATTGC TGATAGTCTC AGGTTCTGTG        200
AGTAACAGCA AAAAAACTTT GTATCTAACT TCAACCAGAG CAGGCTGTAC        250
CCTTAAGCTC T                                                  261
```

We Claim:

1. A method for identifying and isolating a senescence-related gene, which method comprises the steps of:

(a) isolating mRNA from a senescent cell and a young quiescent cell;

(b) amplifying in separate reaction mixtures said mRNA from said senescent cell and said young quiescent cell to produce amplified gene sequences;

(c) separating said amplified gene sequences in each reaction mixture by size and/or charge; and

(d) analyzing said amplified gene sequences separated in step (c) to identify an amplified gene sequence from young quiescent and young dividing cells that is present at a level different from that observed from said senescent cells.

2. The method of claim 1 wherein said amplifying is by a polymerase chain reaction.

3. The method of claim 1 wherein said separating is by gel electrophoresis.

4. The method of Claim 1, wherein said amplifying step comprises the steps of:

(i) aliquoting each different mRNA preparation into at least four and up to twelve different aliquots and adding to each aliquot reverse transcriptase and a primer selected from a group of primers consisting of primers having a 3'-end defined by a sequence $5'-T_{6-12}R_1R_2-3'$, R_1 is A, G, or C; and R_2 is A, G, C, or T, under conditions such that reverse transcriptase-mediated primer extension can occur;

(ii) further subdividing the aliquots prepared in step (1) into at least four and up to twenty different reaction vessels and adding to each vessel DNA polymerase

and a primer selected from a group of primers consisting of primers each having a different 3'-end from other primers in said group, said 3'-end defined by a random sequence of 10 to 14 nucleotides, under conditions such that DNA polymerase-mediated primer extension can occur.

5. The method of Claim 1, wherein said analysis comprises physically removing an amplified gene sequence from the gel and determining its nucleotide sequence, either directly or after cloning into a recombinant DNA vector.

6. The method of Claim 4, wherein said primers are selected from the group consisting of primers in Table 1, above.

7. The method of Claim 6, wherein said DNA polymerase is a thermostable DNA polymerase; step (ii) further comprises extending said primers in a polymerase chain reaction of at least ten cycles of primer annealing, extending, and denaturing; said cycles comprise a first set of two to four cycles of low-temperature primer annealing and a second set of at least six to eight cycles of high-temperature primer annealing; and said low-temperature primer annealing is conducted at a temperature at least 10°C below said high-temperature annealing.

8. A purified oligonucleotide probe comprising a sequence of at least 12 contiguous nucleotides identical or complementary to a contiguous sequence from a nucleic acid in Table 4 or Table 6, above.

9. A method for detecting senescent cells and for distinguishing senescent cells from non-senescent cells, said method comprising the steps of:

(a) contacting mRNA present in a cell or tissue with a labelled nucleic acid probe that comprises a sequence of a senescence-related gene under conditions such that complementary nucleic acids hybridize to one another;

(b) determining whether specific hybridization has occurred; and

(c) correlating the presence of senescent and non-senescent cells with the occurrence of hybridization.

10. The method of Claim 9, wherein said labelled nucleic acid probe comprises a sequence of at least 12 contiguous nucleotides identical or complementary to a contiguous sequence from a nucleic acid in Table 4 or Table 6, above.

11. The method of Claim 10, wherein said mRNA is in a tissue section, and said contacting step is conducted in situ.

12. A method for screening compounds to identify compounds that can alter gene expression in senescent cells, which method comprises:

(a) contacting senescent cells with a compound;

(b) determining mRNA expression patterns in said senescent cells by determining expression levels of mRNA of two or more senescence-related genes; and

(c) correlating an alteration in mRNA expression of a senescence-related gene with a compound that can alter gene expression in senescent cells.

13. The method of Claim 12, wherein said senescence-related genes are selected from the group consisting of genes in Table 2, above.

14. The method of Claim 12, wherein said compounds have, prior to step (a), been tested for ability to modulate activity or expression levels of a first senescence-related gene product and determined to have said ability.

15. The method of Claim 12, wherein step (b) comprises determining expression levels of mRNA of more than two but less than five senescence-related genes.

16. The method of Claim 12, wherein step (b) comprises determining expression levels of mRNA of more than five but less than ten senescence-related genes.

17. The method of Claim 12, wherein step (b) comprises determining expression levels of mRNA of more than ten but less than twenty senescence-related genes.

18. The method of Claim 14, wherein said first senescence-related gene product is beta-galactosidase.

19. The method of Claim 14, wherein said first senescence-related gene product is collagenase.

20. The method of Claim 14, wherein said first senescence-related gene product is IFN gamma.

21. Method for extending the proliferative capability of a population of cells from a donor comprising the steps of:

a) isolating cells from said donor;

b) separating senescent cells from young cells; and,

c) reintroducing said young cells into said donor.

22. The method of claim 21 wherein said method further comprises, after step b), expanding said young cells in the presence of an agent which slows the replicative senescence of said cells.

23. A method for destroying a cell expressing a senescence-related gene product, wherein said method comprises the step of providing a toxic substance which specifically destroys said cell..

24. The method of claim 23 wherein said toxic substance is a toxic substrate, wherein said toxic substrate is activated by a senescence-related gene product.

25. The method of claim 24 wherein said toxic substate is activated by beta-galactosidase.

25. A method for destroying a cell expressing a senescence-related gene product wherein said method comprises the step of providing a antibody to said senecence-related gene product, wherein said antibody is linked with a toxic substance.

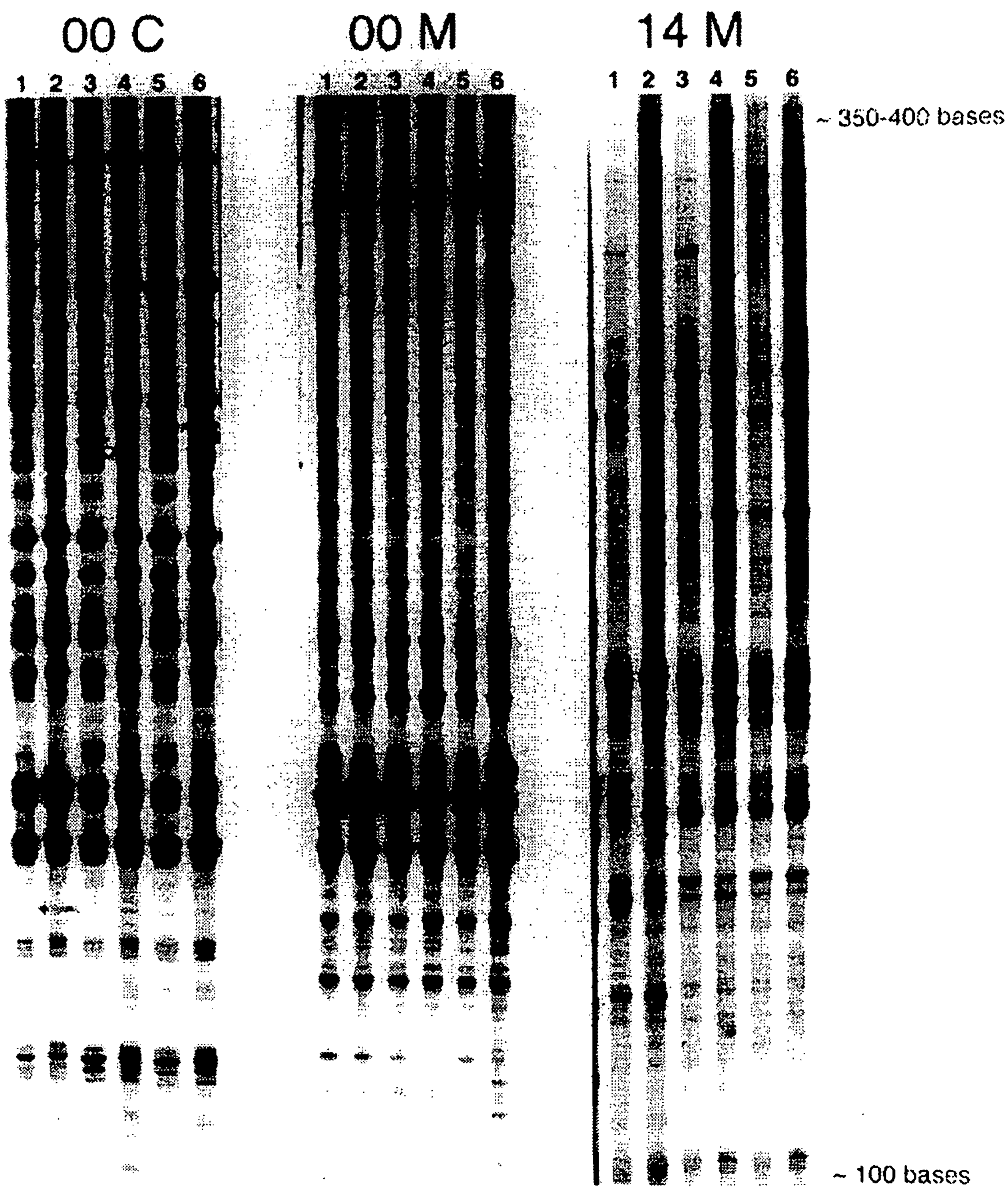


FIG. 1.

FIG. 2

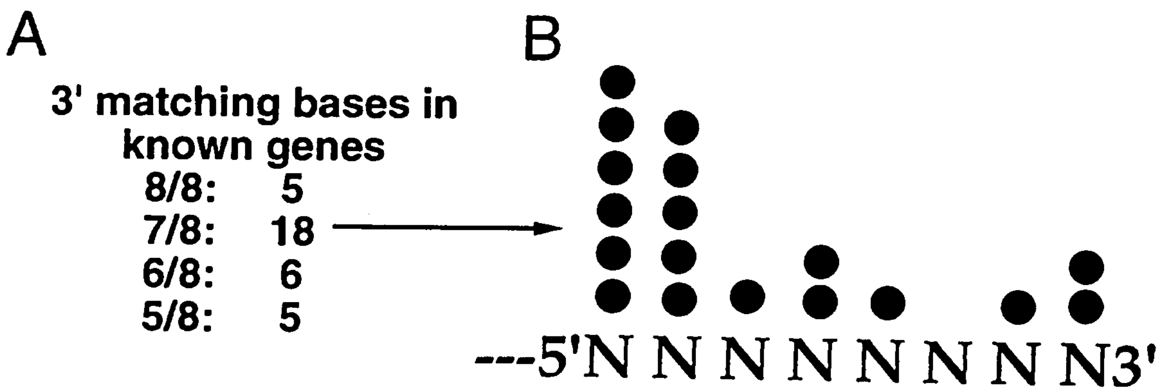
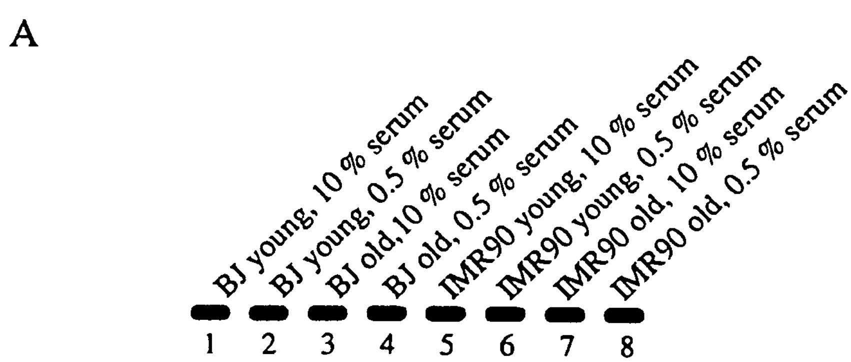

A
3' matching bases in known genes
8/8: 5
7/8: 18
6/8: 6
5/8: 5
B
---5'N N N N N N N N3'

FIG. 4a.


A
BJ young, 10 % serum
BJ young, 0.5 % serum
BJ old,10 % serum
BJ old, 0.5 % serum
IMR90 young, 10 % serum
IMR90 young, 0.5 % serum
IMR90 old, 10 % serum
IMR90 old, 0.5 % serum
1 2 3 4 5 6 7 8

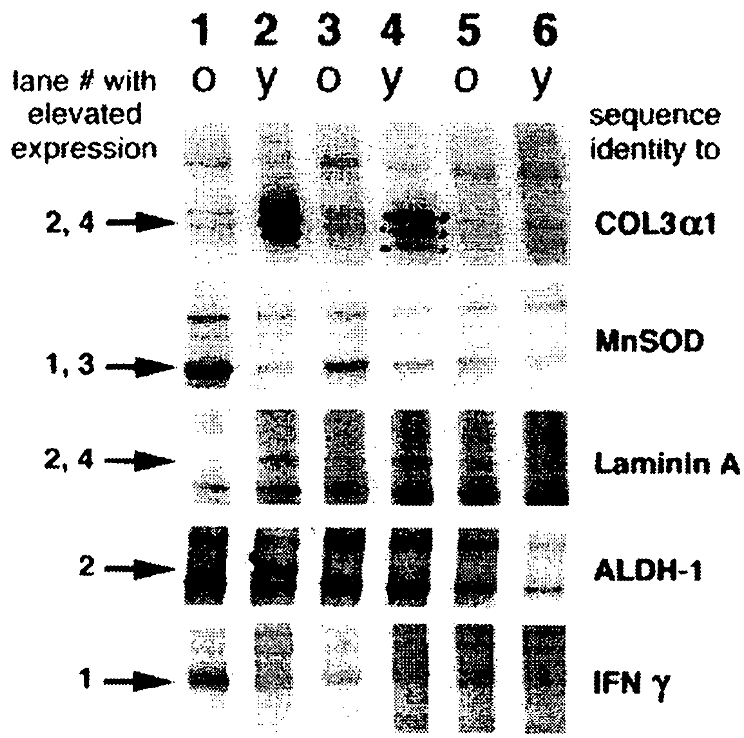

1 2 3 4 5 6
lane # with elevated expression
o y o y o y
sequence identity to
2, 4
COL3α1
MnSOD
1, 3
2, 4
Laminin A
2
ALDH-1
1
IFN γ

FIG. 3.

FIG. 4b.

B. PREVIOUSLY IDENTIFIED CHANGES IN GENE EXPRESSION DURING SENESCENCE

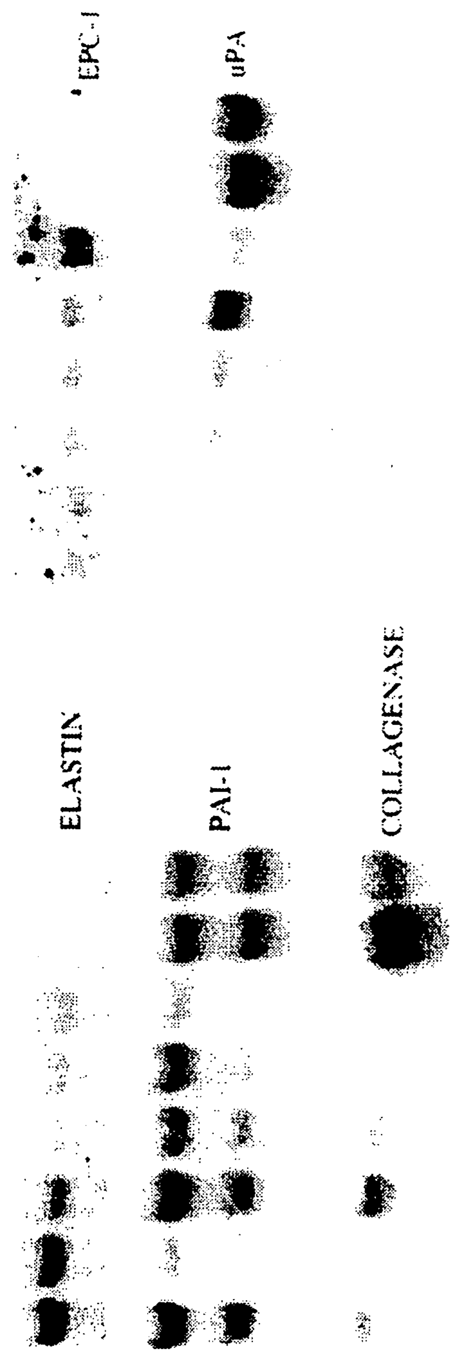


FIG. 4C.

C. Known young- and senescent-specific genes from EDD

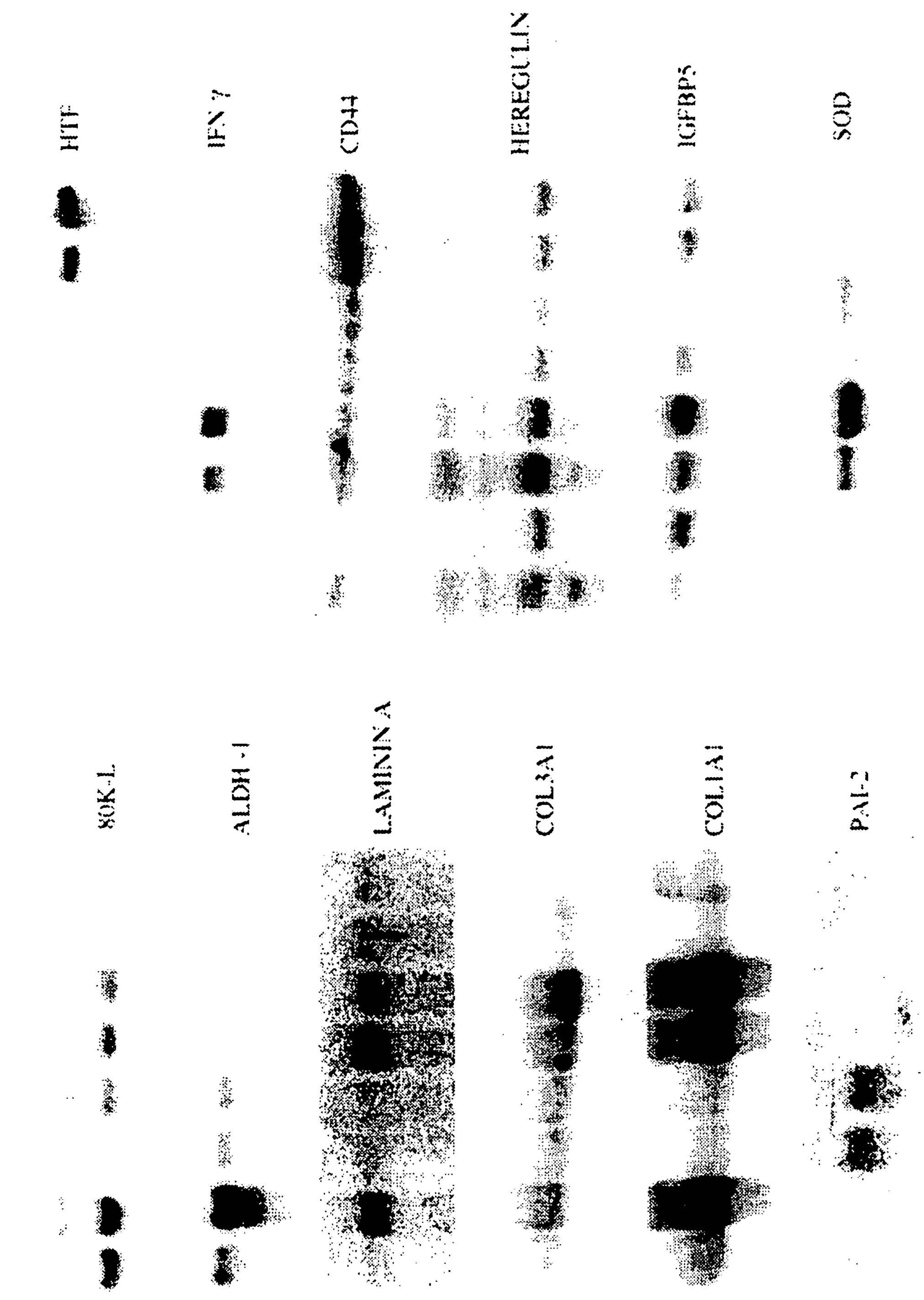


FIG. 4d.

D. Novel young- and senescent-specific genes from EDD